DESIGN, DEVELOPMENT AND CHARACTERIZATION OF AN ANTI-NEOPLASTIC INJECTION BY USING LYOPHILIZATION TECHNIQUE

Dissertation submitted to

THE TAMILNADU Dr. M.G.R. MEDICAL UNIVERSITY, CHENNAI-32

In partial fulfillment for the award of the degree of

MASTER OF PHARMACY

IN

PHARMACEUTICS

Submitted by

Register Number: 261210005

UNDER THE GUIDANCE

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CERTIFICATE

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DECLARATION

I hereby declare that the thesis entitled **"DESIGN, DEVELOPMENT AND CHARACTERIZATION OF AN ANTI-NEOPLASTIC INJECTION BY USING LYOPHILIZATION TECHNIQUE "** has been originally carried out by me under the supervision and guidance of **Mr. UDAY CHAND, Mpharm.,** (Industrial Guide) & **Dr.Rama,M.Pharm.,Ph.D.** (Institutional Guide) Asst.Professor, Department of Pharmaceutics, C.L.Baid Metha college of Pharmacy, Chennai-97, during the academic year 2013-2014.

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G. DHARANI SAI

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ABBREVIATIONS

FDA	-	Food and Drug Administration
°C	-	Degree centigrade
KPa	-	Kilo pascals
Nm^{-2}	-	Newton per meter square
Hg	-	Mercury
Tg	-	Glass transition temperature
Teu	-	Eutectic temperature
Tc	-	Collapse temperature
Tp	-	Product temperature
Ts	-	Shelf temperature
Po	-	Vapour pressure of ice
Pc	-	Chamber pressure
KF	-	Karl fisher
TGA	-	Thermal gravimetric analysis
IPEC	-	International pharmaceutical Excipients council
USP	-	United states pharmacopeia
WFI	-	Water for injection
API	-	Active pharmaceutical ingredient
EMEA	-	European medicines agency
WHO	-	World health organisation
IIG	-	Inactive ingredient guide
PIL	-	Patient information Leaflet
BP	-	British pharmacopeia
PDR	-	Physician’s Desk Reference
I.V	-	Intravenous
PAT	-	Process analytical technology
GMP	-	Good manufacturing practice
CAS	-	Chemical Abstract Service

HPLC	-	High pressure liquid chromatography
SS	-	Stain less steel
PVDF	-	Poly vinylidenedifluoride
GR	-	Guaranteed Reagent
RH	-	Relative humidity
BM	-	Bendamustine hydrochloride
TAB	-	Tertiary butyl alcohol
CCs	-	Clear colourless solution
ND	-	Not detected
BEND-IV	-	Bendamustine IV
IPA	-	Isopropyl acetate

NOMENCLATURE

Mg	-	Milligram
Mm	-	Milli meter
µm	-	Micro meter
Min	-	Minute
w/v	-	Weight by volume
mOsm	-	Milliosmoles
%	-	Percentage
w/w	-	Weight by weight
q.s	-	Quantity sufficient
ml	-	Millilitre

ISO 9001 : 2008 Certified

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1. INTRODUCTION

1.1 INTRODUCTION OF PARENTERALS[1]

The parenteral administration route is the most effective and common form of delivery for active drug substance with poor bio availability and the drugs with a narrow therapeutic index. These products are intended for administration by injection.

1.1.1 Types of Parenteral

Parenterals are broadly classified into three types

a) Small volume parenterals(SVP)

All the sterile products packaged in vials, ampoules, cartridges, syringes bottles or other container that is 100 ml or less fall under the class of SVP

b) Large volume parenterals(LVP)

The USP provides the definition for large volume parenteral (LVP) " where used in the pharmacopeia, the designation large volume solutions applies to an injection that is intended for intravenous use and is packaged in containers holding 100 ml or more". LVP'S means a terminally sterilized aqueous drug product packaged in a single dose container with a capacity of 100 ml or more and intended to be administered or used in humans. It includes IV infusions, irrigation solutions, peritoneal dialysates and blood collecting units with anticoagulants.

1.1.2 Route of Administration[1]

Figure 1

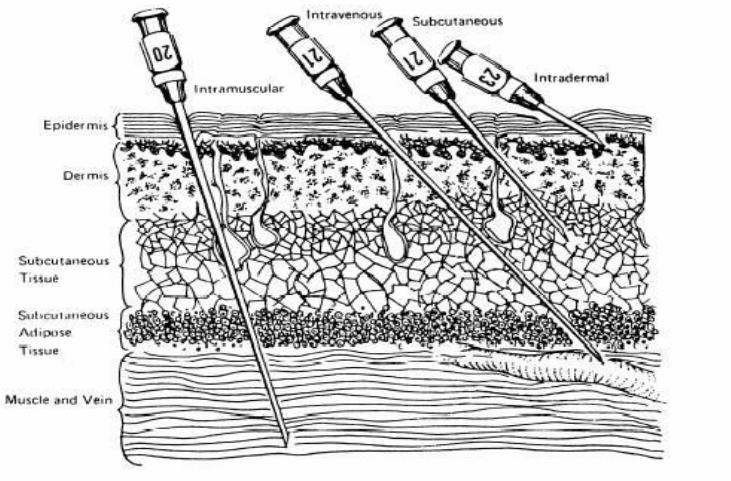


Table 1

Route	Injection site
Intravenous (IV)	Vein
Intramuscular (IM)	Muscle tissue
Intradermal (ID)	Dermis of the skin
Subcutaneous (subcut: SQ)	Subcutaneous tissue of the skin
Intrathecal (IT)	Subarachnoid space of the spinal cord
Epidural	Epidural space of the spinal cord
Intra-arterial	Artery
Intra- articular	Joint space
Intracardiac	Heart
Intraocular	Eye
Intraperitoneal	Peritoneal cavity

1.1.3 Advantages

- Provides drug and nutritional options for patients unable to tolerance oral therapy.

- Circumvents absorption limitations of gastrointestinal tract.
- Quick onset of action.
- Localized delivery.
- Prolonged duration of effect.

1.1.4 Disadvantages

- Difficulty/ impossibility of drug removal /reversal.
- Risk of infection.
- Risk of emboli.
- Risk of hypersensitivity reactions.
- Higher costs.

1.2 LYOPHILIZATION[2]

Nowadays, in the pharmaceutical field, there is a great number of substances which need to be stored in a dry state due to their instability in the presence of water, for example, the antibiotics, vaccines, peptides and proteins. Lyophilization or Freeze-Drying, fills an important need in pharmaceutical manufacturing technology by allowing drying of heat-sensitive drugs and biological at low temperature under conditions that allow removal of water by sublimation, or a change of phase from solid to vapour without passing through the liquid phase. While the most common application of pharmaceutical freeze-drying is in the production of injectable dosage forms, the process is also used in the production of diagnostics and, occasionally, for oral solid dosage forms where a very fast dissolution rate is desired.[1] About 50% of the currently marketed biopharmaceuticals are lyophilized, representing the most common formulation strategy.[2]

1.3 DEFINITION OF LYOPHILIZATION[3-9]

In simple terms, Lyophilization or Freeze-Drying is a unit operation in which water or solvent is removed from a product after it is frozen and placed under

a vacuum, allowing the ice to change directly from solid phase to vapor without passing through a liquid phase (sublimation).In this process, the moisture content of the product is reduced to such a low level that does not support biological growth or chemical reactions.

1.4 PRINCIPLE OF LYOPHILIZATION

The main principle involved in Lyophilization is a phenomenon called Sublimation, where water passes directly from solid state (ice) to the vapor state without passing through the liquid state.

The phase diagram of water show that two phases coexist along a line under the given conditions of temperature and pressure, while at the triple point (0.0075 °C at 0.61kPa or 610 Nm^{-2}; 0.01 °C at 0.00603 atm; 0.0099°C and 4.579 mm of Hg), all three phases coexist.

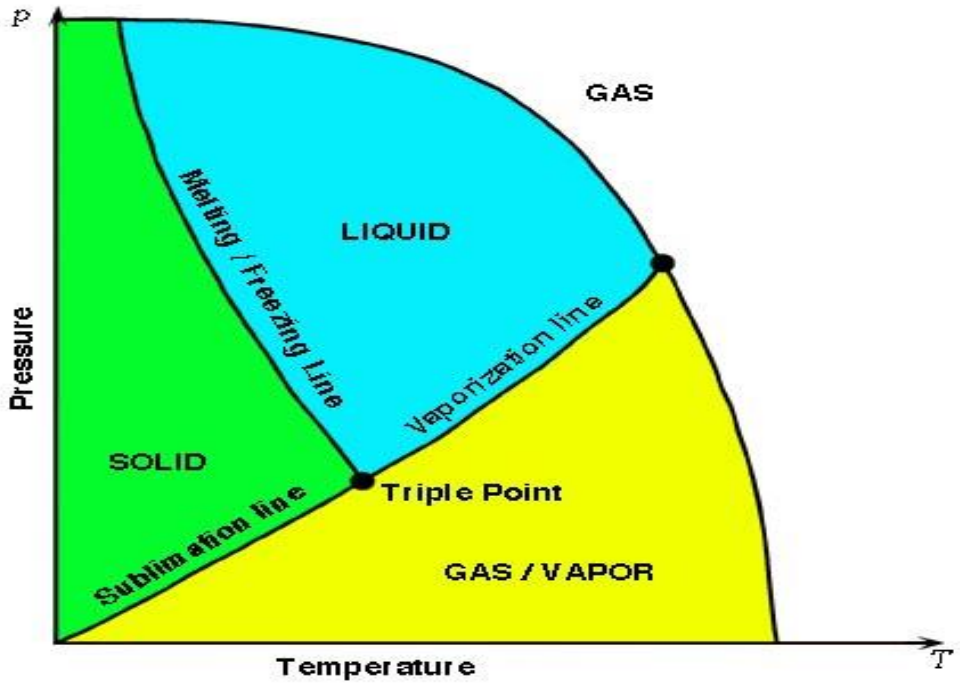


Figure 2 Phase diagram showing the triple point of water

Lyophilization is performed at temperature and pressure conditions below the triple point, to enable sublimation of ice. The entire process is performed at low temperature and pressure, hence is suited for drying of thermolabile compounds. LYO lab lyophilizer is used for present study.

Figure 3 LYO lab Lyophilizer used for the present study

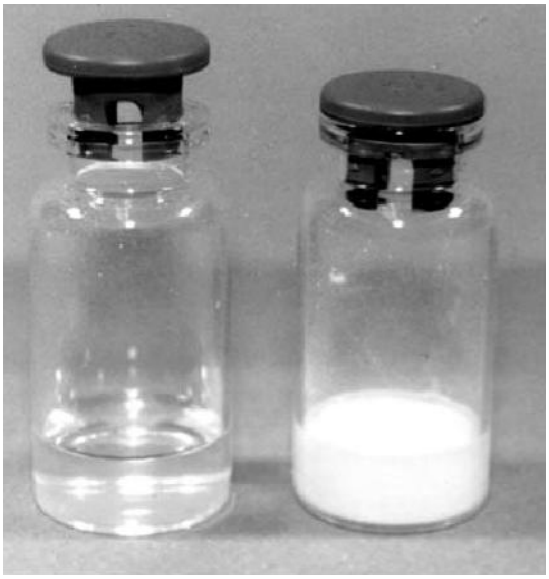


Figure 4 Vials typically used for Lyophilization showing slotted stopperIn the open and closed positions

1.5 STAGES OF FREEZE DRYING[10]

A typical freeze-drying process consists of three separate, unique and inter-dependent processes namely

- Freezing (solidification)
- Primary drying (sublimation)
- Secondary drying (desorption)

1.5.1 Freezing

Freezing is generally the first step in a freeze-drying process, in which nearly 90% of the water is converted to ice crystals while all solutes in the formulation are solidified into a matrix either in amorphous or crystalline state, or in a mixture. The conversion from water to ice crystals starts with ice nucleation, which is followed by ice crystal growth. For a typical pharmaceutical formulation, the ice nucleation temperature is often in the range of 10–15ºC or more below the equilibrium freezing point; a phenomenon referred to as Super-Cooling.

Products freeze in two ways, depending on the makeup of the product. The majority of products that are subjected to freeze drying consist primarily of water, the solvent, and the materials dissolved or suspended in the water, the solute.It is very important in freeze drying to refreeze the product to below the eutectic temperature before beginning the freeze drying process. Small pockets of unfrozen material remaining in the product expand and compromise the structural stability of the freeze dried product.

The second type of frozen product is a suspension that undergoes glass formationduring the freezing process. Instead of forming eutectics, the entire suspension becomes increasingly viscous as the temperature is lowered. Finally the product freezes at the glass transition pointforming a vitreous solid. This type of product is extremely difficult to freeze dry.[10]

a) Process Design and Control [6]

During freezing, the chamber pressure is slightly lower than the atmospheric pressure due to low temperature, or/and pre reduction of chamber pressure to enhance the sealing of the chamber door. The stage of the process is generally controlled by the shelf-cooling/heating rate, shelf-holding temperatures, and holding times.

b) Pre freezing hold

In order to facilitate relatively uniform ice nucleation and ice crystal growth, the product vials on the shelf are held at a temperature lower than room temperature before cooling down. This temperature is generally the loading temperature, for example, 5ºC. For formulations with high super-cooling temperature, holding at an even lower temperature (a few degrees higher than the ice nucleation temperate) is more appropriate.

c) Cooling Down to the Final Freezing Temperature

Cooling the product to a terminal (final) freezing temperature facilitates the ice nucleation/growth and solute solidification. If a super-cooling hold is applied, a relatively faster cooling is generally helpful for intra-vial uniformity of ice formation.

d) Annealing[11]

Annealing is simply holding the product at a temperature above the final freezing temperature for a defined period to crystallize the potentially crystalline components (usually, crystalline bulking agent) in the formulation during the freezing stage. An annealing step is frequently necessary to allow efficient crystallization of the crystalline bulking agent, such as mannitol or glycine. Failure to crystallize the bulking agent if bulking agent crystallizes during primary drying, vial breakage may result, which is common if a high fill depth of concentrated mannitol is used.Vial breakage can be prevented by crystallization of

mannitolduring freezing using slow freezing or by avoiding a temperature lower than about −25°C until the mannitol has completely crystallized. Completion of crystallization may be facilitated by annealing. After annealing, the product temperature is generally lowered to a final temperature and held long enough to complete solidification.

1.5.2 Primary Drying [10]

After freezing, the productis ‘‘dried’’ at relatively low temperature and low pressure in which ice can be removed from the frozen product via sublimation, resulting in a dry, structurally intact product.

This requires very carefully control of the two parameters,

1) Temperature and

2) Pressure involved in freeze-drying system.

It is extremely important that the temperature at which a product is freeze-dried is balanced between the temperature that maintains the frozen integrity of the product and the temperature that maximizes the vapor pressure of the product. This balance is key to optimum drying.

Energy supplied to sublime a gram of water from the frozen to the gaseous state as is (2700 joules per gram of ice).

1.5.2.1 Process design principle

a) Heat Transfer

Heat transfer in lyophilization can occur by three processes: conduction, convection and radiation. Of these, Conduction is the main contributor to the heat transfer.

Conduction can be modeled by Fourier's law:

$$\frac{dQ}{dt} = A\lambda \frac{dT}{dz}$$

Where the heat flow is dQ/dt, A is the area of the surface, λ is the thermal conductivity of the material and $dT$is the temperature gradient across the thickness of the material dz.

Radiation heat transfer must also be taken into account in lyophilization. This is the transfer of heat by electromagnetic radiation. A real body emits and absorbs radiation according to the equation:

$$Q_{rad} = Ae\sigma T^4$$

Where e = emissivity, σ = Stefan-Boltzmann constant and T = Absolute Temperature.

b) Mass Transfer [11]

The rate at which the ice sublimes will be affected by the resistances that it encounters. The heat and mass transfer causes the top of the product to dry first with drying proceeding downward to the bottom of the vial. Therefore, as drying proceeds, there exists a three component or layer system in each vial – the upper dry product, the middle sublimation front, and the lower frozen liquid product. As the dried layer increases, it becomes a great barrier

Or the source of greatest resistance to the transfer of mass out of the vials.

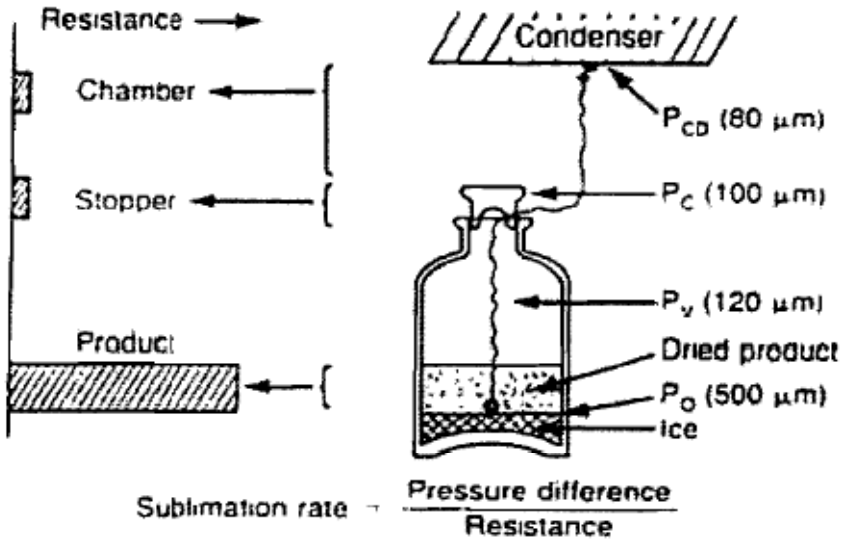


Figure 5 Resistances and their relative contributions in Mass Transfer

1.5.2.2 Process design and control[12]

a) Target Product Temperature and Structural Collapse

- Collapse temperature for an amorphous system refers to the temperature, above which the dried region adjacent to ice loses its structure,that is; collapse temperature (T_c) can be higher than the glass transition temperature (T_g).

- A safety margin should be kept during primary drying, that is, the product temperature (T_p) should be 2–5ºC below T_c or T_e. This safety margin accounts for (i) the product temperature increases, in general, between 1 and 3ºC, due to the increase of resistance from the dried-layer as drying progresses; (ii) the heterogeneity of product temperature across the shelf and from shelf to shelf.

b) Chamber Pressure

- A most efficient primary drying condition for the product in a given vial should be a combination of a ‘‘very high’’ shelf temperature with a ‘‘very low’’ chamber pressure.

- The chamber pressure is not generally lower than 50 mTorr.

- As a general rule, a chamber pressure, approximately between 10% and 50% of ice vapor pressure at the target product temperature, which generally falls into a range between 50 and 200 mTorr is chosen for primary drying. This moderate chamber pressure (100–150 mTorr) gives optimal homogeneity of heat transfer in a set of vials.[25] Therefore; the optimum chamber pressure is a compromise between high sublimation rate and homogenous heat transfer.

- The optimal chamber pressure at known target product temperature (T_p) can be given by the equation

$$\mathbf{P_c = 0.29 \times 10^{(0.019 \times T_p)}}$$

Where P_c is chamber pressure (Torr) and T_p is product temperature (°C).

c) Shelf Temperature

- Shelf temperature can be determined experimentally by manometric temperature measurement (MTM),[12]
- The shelf temperature is higher than the product temperature and sometimes can be much higher, up to 40ºC.

1.5.2.3 Process monitoring and control [6]

a) Determination of End Point of Primary Drying

Once the shelf temperature and chamber pressure for primary drying are determined, the primary drying process, essentially including two steps

b) Ramp from Freezing to Primary Drying:

After evacuation to reduce the chamber pressure to the target level, the shelf temperature is ramped up to the target value. The ramp rate should not be too

high, normally less than 1ºC/min. During this initial period of sublimation, ice sublimation rate can be quite high, since the resistance in the product is nearly zero.

c) Duration of Primary Drying:

The duration of primary drying is determined by the ice sublimation rate, the characteristics of formulation solution and can be roughly estimated

- Theoretically by calculations based upon the mass and heat transfer equations.
- In practice, the duration is determined by monitoring the drying progression.

1.5.3 Secondary Drying

When all ice crystals are removed from the product by sublimation, the dried product contains a fairly high amount of ‘‘unfrozen water’’ (5–20% in the solid content). In the secondary drying stage, the unfrozen water is further reduced to a desired, much lower level at a higher temperature.

The glass transition temperature (T_g) of the dried formulation is a function of the moisture content, which is governed by the Gorden-Taylor equation. Therefore, the T_g changes sharply with the decrease of moisture during the ramp from primary drying to secondary drying, and during secondary drying.

1.5.3.1 Process design and control[13]

a) Heating Rate and Chamber Pressure

- Because of the fairly high residual moisture content in the amorphous product early in secondary drying and, thus, low glass transition temperature, the potential for collapse is greatest early in secondary drying. So a ramp rate of 0.1 or 0.15°C/min for amorphous products is generally safe and appropriate.

- Crystalline products do not have any potential for collapse during secondary drying, and a higher ramp rate is suggested for such products (0.3 or 0.4°C/min).

- The chamber pressure in primary drying is also appropriate for secondary drying, it is not necessary to change chamber pressure for secondary drying.

b) The Shelf Temperature and Secondary Drying Time[14]

- The products should be kept at "high" temperature for a period sufficient to allow the desired water desorption. Usually, it is better to run a high shelf temperature for a short time than a low temperature for a long period.

- Amorphous products are more difficult to dry than crystalline products. Thus, higher temperatures and longer times are needed to remove the absorbed water.

- The secondary drying conditions also depend on the solute concentration. At higher solute concentration (i.e., >10% solids in solution), the dry product has smaller specific area, and it is more difficult to remove the absorbed water; thus longer times and/or higher temperatures are needed to finish secondary drying.

- Normally, drying times of 4–10 h at the range 40 to 50°C.

- The optimum secondary drying time can be determined by real-time residual moisture measurement using Karl Fischer titration (KF), thermal gravimetric analysis (TGA), or near IR spectroscopy.[15]

The product is usually freeze dried to very low residual moisture content (about 0.5%). Usually, a combination of long drying times (6 h) and low shelf temperature (about 0°C) are best, but the exact conditions must be determined by trial and error.

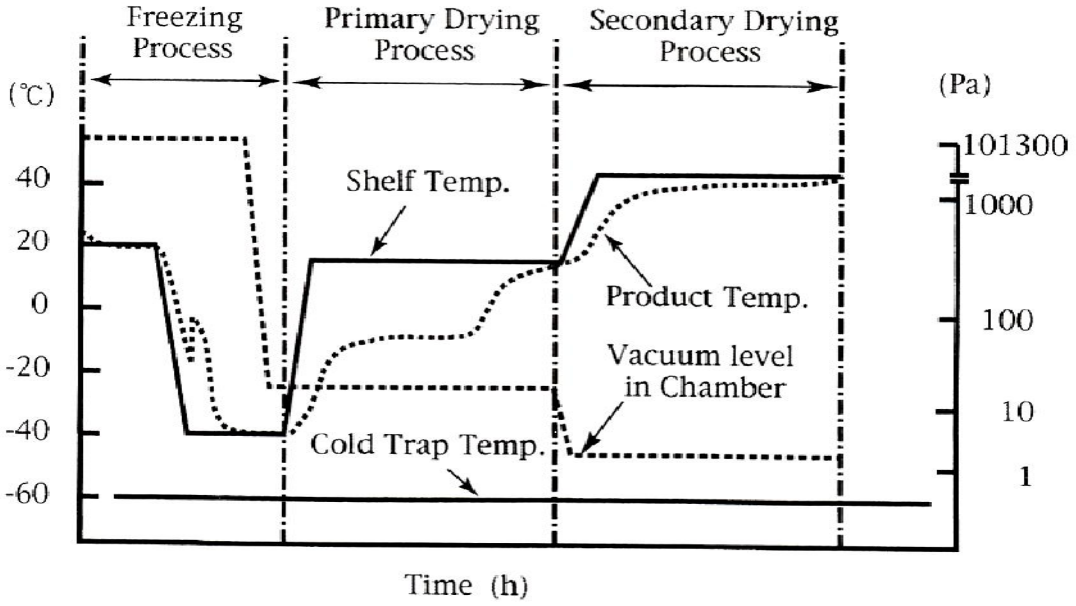
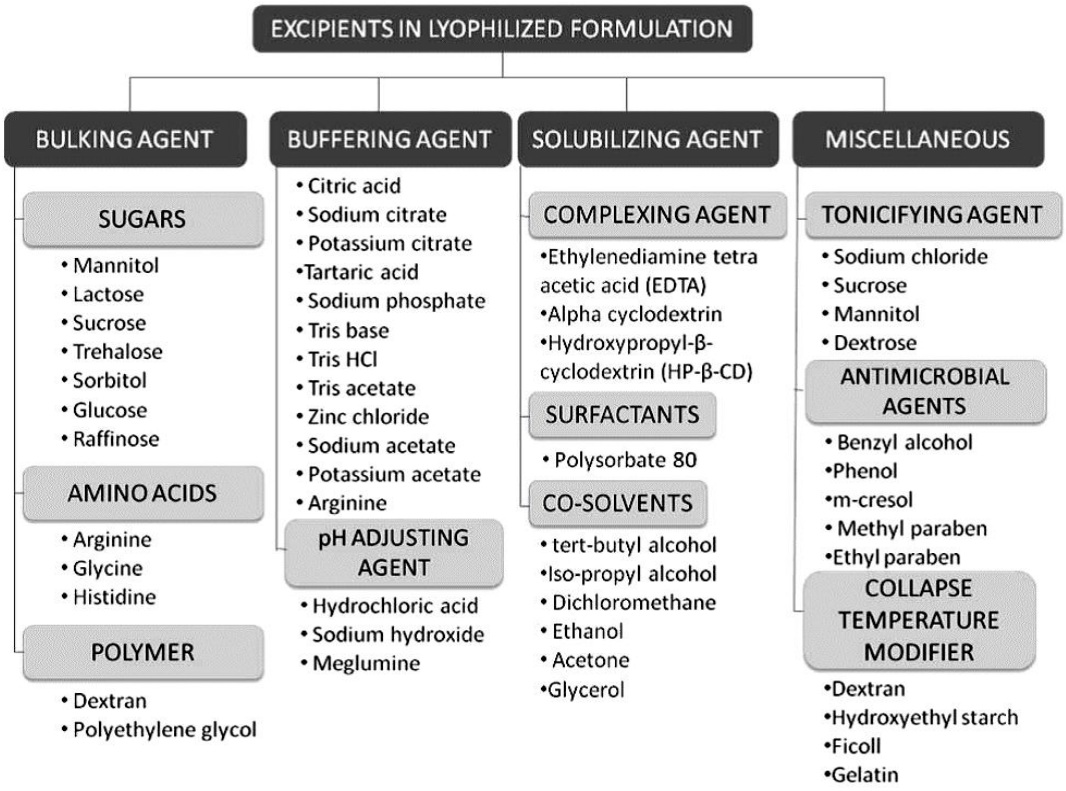


Figure 6 Plot of process variables such as shelf temperature, product temperature and vacuum level in chamber during freeze-drying cycle.

1.6 EXCIPIENTS USED IN LYOPHILIZATION[16]

The International Pharmaceutical Excipients Council (IPEC) has defined excipients as: **"…substances other than thepharmacologically active drug or prodrug which areincluded in the manufacturing process or are containedin a finished pharmaceutical product dosage form."**

- **The excipients commonly used in the lyophilization have been classified as**:



a) Bulking agent[17-19]

- Bulking agents, as the name implies, form the bulk of the lyophilized product and provide an adequate structure to the cake. These are generally used for low dose (high potency) drugs that do not have the necessary bulk to support their own structure. These are particularly more important when the total solid content is less than 2%.In such cases, a bulking agent is added to the formulation matrix.

- Mannitol and glycine, are the most commonly used bulking agents, followed by glucose, sucrose, lactose, trehalose and dextran.

b) Co-Solvents[20]

- Water is the most commonly used solvent for lyophilization. However, organic solvents are sometimes used to increase the primary drying rate by increasing the sublimation rates, improve product stability, decrease reconstitution time by improving drug wettability or solubility, and also enhance the sterility assurance of the sample solution.

- The most commonly used solvent is a tertiary-butanol/water combination.

Liophilization can be applied for

- Non – biological where the process is used to dehydrate or concentrate reactive or heat labile chemicals

- Non-living bioproducts

  - Enzymes
  - Hormones
  - Antibodies
  - Inactivated vaccines

- Industrially useful bio products

1.7 DESIRED CHARACTERISTICS OF FREEZE-DRIED PRODUCTS[6, 11]

The desired characteristics of a freeze-dried pharmaceutical dosage form include:

- A freeze dried product is expected to have nearly full recovery of the original chemical or biological potency after reconstitution

- Cake should be intact occupying the same shape and size as the original frozen mass
- Have sufficient mechanical strength to prevent cracking, powdering or collapse
- Uniform colour and pharmaceutically elegant appearance
- The product should be sufficiently dry to maintain stability and sufficiently porous which leads to rapid and complete dissolution
- The product should be free from microorganisms, pyrogens and particulates

The desired characteristics can be achieved by proper formulation of the product and by employing optimum freeze-drying cycles.

1.8 ADVANTAGES OF LYOPHILIZATION [7, 11]

The principle advantages of lyophilization includes

- Minimum damage and loss of activity in delicate heat-labile materials
- Removal of water without excessive heating of the product
- Enhanced product stability in a dry state
- Rapid and easy dissolution of reconstituted product due to the porous nature of the product
- Good for oxygen and/or air-sensitive drugs
- Sterility of product can be achieved and maintained
- Constituents of the dried material remain homogenously dispersed
- Reduced weight of the product which makes the product easier to transport

1.9 DISADVANTAGES OF LYOPHILIZATION [7, 20]

- Increased handling and processing time
- Need for sterile diluent upon reconstitution
- Cost and complexity of equipment

1.10 FINISHED PRODUCT INSPECTION - MELTBACK

The USP points out that it is good pharmaceutical practice to perform 100% inspection of parenteral products. This includes sterile lyophilized powders.

1) Melt back is a form of cake collapse and is caused by the change from the solid to liquid state. That is, there is incomplete sublimation (change from the solid to vapor state) in the vial. Associated with this problem is a change in the physical form of the drug substance and/or a pocket of moisture. These may result in greater instability and increased product degradation.

2) Another problem may be poor solubility. Increased time for reconstitution at the user stage may result in partial loss of potency if the drug is not completely dissolved, since it is common to use in-line filters during administration to the patient.

Manufacturers should be aware of the stability of lyophilized products which exhibit partial or complete meltback. Literature shows that for some products, such as the cephalosporins, that the crystalline form is more stable than the amorphous form of lyophilized product. The amorphous form may exist in the "meltback" portion of the cake where there is incomplete sublimation

Table 2Marketed lyophilized formulations[10]

DRUG	CATEGORY	MARKETED NAME	MANUFACTURER
Amifostine	Cytoprotective agent	Ethyol®	MedImmune Oncology
Amphotericin B	Antifungal	Ambisome®	Astellas
Acyclovir sodium	Antiviral	Zovirax®	GlaxoWellcome
Azithromycin	Antibiotic	Zithromax®	Pfizer
Cefazolin sodium	Antibiotic	Ancef ®	GlaxoSmith-Kline
Chlorothiazide sodium	Diuretic and hypertensive	Diuril®	Merck
Cisplatin	Antineoplastic	Platinol®	Bristol Myers Oncology
Dantrolene sodium	Muscle relaxant	Dantrium®	Procter & Gamble
DaunorubicinHCl	Antibiotic	Cerubidine®	Bedford
Diltiazem	Antianginal	Cardizem®	Hoechst Marion Roussel
Doxorubicin HCl	Antineoplastic	Rubex®	Bristol Myers Squibb
Ganciclovir sodium	Treatment of CMV retinitis in immunocompromized patient	Cytovene®	Roche
HydromorphoneHCl	Opioid analgesic	Dilaudid-HP®	Abbott
Lansoprazole	Proton pump inhibitor	Prevacid®	TAP
Metronidazole	Antibacterial	Flagyl®	Pfizer
Mitomycin	Antineoplastic	Mutramycin®	Bristol Myers Squibb
Pentostatin	Antineoplastic	Nipent®	Supergen
Phentolaminemesylate	Antihypertensive	Regitine®	Novartis
Pralidoxime chloride	Antidote for overdose due to anticholinesterase	Protopam®	Baxter Healthcare
Tazobactam sodium and Piperacillin sodium	Antibacterial combination	Zosyn®	Lederle
Tigecycline	Antibacterial	Tygacil®	Wyeth
VancomycinHCl	Antibiotic	VancocinHCl®	Eli Lilly
Vecuronium bromide	Muscle relaxant	Norcuron®	Organon

2. LITERATURE REVIEW

Julia CK *et al.*, (2011)explained that Lyophilization is a drying process in which freezing step is an important step, that impacts both process performance and product quality. In this review, the physico-chemical fundamentals of freezing are first summarized. The available techniques that can be used to manipulate or directly control the freezing process in lyophilization are also reviewed. It aims to provide an awareness of not only the importance but also the complexity of the freezing step in lyophilization and its impact on quality attributes of biopharmaceuticals and process performance. With a deeper understanding of freezing and the possibility to directly control or at least manipulate the freezing behavior, more efficient lyophilization cycles can be developed and the quality and stability of lyophilized biopharmaceuticals can be improved.[21]

Nishant T*et al.*, (2010)explained about the Background of Bendamustine (Treanda, Ribomustine), as a water-soluble, bifunctional chemotherapeutic agent that also has potential antimetabolite properties. He also stated that the Bendamustine has been designated as an orphan drug in the United States, conferring prolonged market exclusivity. This article provides a comprehensive review of the data on efficacy and toxicity from trials investigating the use of bendamustine for the treatment of lymphoproliferative neoplasms.[22]

Nazik E*et al.*, (2010)explained that Flutamide (FLT), an anticancer drug for prostatic carcinoma, has poor aqueous solubility and low oral bioavailability. The present research describes the ability of β-cyclodextrins(β-CD) and hydroxypropyl-β-cyclodextrin (HP-βCD) to form complexes with Flutamide with enhanced solubility and dissolution rate in vitro. FLT–CD lyophilized dispersions (LDs) were prepared via Lyophilization monophase solution technique using tertiary butyl alcohol (TBA) as a co solvent. This shows an AL-type phase solubility diagram consistent with a linear increase in drug solubility as a function of CD concentration. Based on the data from differential scanning calorimetry (DSC) and X-ray diffractometry (XRD), FLT was fully amorphous in 1:5 FLT-HP-β-CD LD as

indicated by complete disappearance of FLT endothermic and diffraction peaks. The Fourier transform infrared (FTIR) spectra indicated that a FLT–CD interaction took place in the lyophilized complex. These data suggest that cyclodextrins might be useful adjuncts in preparation of immediate-release formulations of FLT.[23]

Meister E *et al.*, (2006) studied the dependency of a collapse temperature (Tc) on total solid content by evaluating physical properties (e.g. viscosity) of selected excipients solutions at 0°C. Thus, to gain a better understanding of collapse behavior and therefore the opportunity to further optimize formulations and freeze drying cycles and to evaluate the transferability of collapse temperatures measured by Freeze Drying Microscopy (FDM) on freeze drying processes, and to establish a general relationship (guideline) between the onset of collapse as detected by microscopy and actual (micro) collapse of a structure in a vial during a freeze drying cycle.[24]

Shailaja R*et al.*, (2005) the purpose of the present studies was to investigate the effect of shelf temperature during primary drying and secondary drying on the degree of cake shrinkage. Freeze drying experiments are performed using 5%w/v sucrose where the drying protocols are altered in order to produce differing product temperature profile. Resistance data during freeze-drying are evaluated by the Manometric Temperature Measurement (MTM) Method. Theoretical simulation of the freeze drying process is performed using the passage freeze-drying Software. The difference between the glass transition temperature and product temperature (Tg-T) obtained from the theoretical analysis is calculated and used for correlation with experimental shrinkage data. Conclusion of experiment is maintained well below the collapse temperature and below glass transition temperature throughout the drying process which is important for prevention of shrinkage.[25]

Jennings TA*et al.*,(2005)examines means for managing the risk that the moisture from elastomer closures may have in producing poor lyophilized products. Assessment of the risk will be based on the frequency distribution of the capacitance of the closures. The importance that the sample size will have on confidence interval

and its effect on managing the risk will be examined. Other key factors that must be taken into account are post lyophilization treatment of the closures and mass of the lyophilized cake. Sound management of the risk of a poor lyophilized product from the residual moisture of closures requires a reliable data base.[26]

Searles JA*et al.*,(2004) performed research on annealing to optimize the primary drying rate, reduce freezing-induced drying rate heterogeneity, and determined Tg in pharmaceutical lyophilization.[27]

Rambhatla S*et al.*, (2004)studied heat and mass transfer scale-up issues during freeze drying: control and characterization of the degree of super cooling.[28]

Tsinontides SC*et al.*, (2004)showed thatFreeze Drying involves transfer of heat and mass to and from the product under preparation, respectively, thus it is necessary to scale these transport phenomena appropriately from pilot plant to manufacturing-scale units to maintain product quality attributes. It also describes the principal approach and tools utilized to successfully transfer the lyophilization process of a labile pharmaceutical product from pilot plant to manufacturing. Based on pilot plant data, the lyophilization cycle is tested during limited scale-up trials in manufacturing to identify parameter set-point values and test process parameter ranges. The limited data from manufacturing are then used in a single-vial mathematical model to determine manufacturing lyophilizer heat transfer coefficients, and subsequently evaluate the cycle robustness at scale-up operating conditions. The lyophilization cycle is then successfully demonstrated at target parameter set-point values.[29]

Edinara AB *et al.*, (2004)described thatFreeze drying is a separation process based on the sublimation phenomenon. This process has the following advantages compared to the conventional drying process. The material structure is maintained, moisture is removed at low temperature (reduced transport rates), product stability during the storage is increased, and the fast transition of the moisturized product to be dehydrated minimizes several degradation reactions. In order to put it to practice, a mathematical model based on fundamental mass and

energy balance equations has been developed, based on a deterministic mathematical model proposed by Liapis and Sadikoglu [Drying Technol.(1997) 791], and used to calculate the amount of removed water and amount of residual water. The optimization procedure showed to be an important tool to improve the process performance since lower energy consumption and hence lower cost has been achieved to obtain the product with the same quality.[30]

Vikas KS *et al.*, (2004)studied the systematical investigation of protein-Mannitol interactions using vacuum drying, to obtain a better understanding of the effect of protein/Mannitol w/w ratios on the physical state of Mannitol and protein secondary structure in the dried state. Solutions containing β-lacto globulin (βLg): Mannitol (1:1-1:15 wt/wt) are vacuum dried at 5°C under 3000 mTorr pressure. The physical state of Mannitol is studied using x-ray powder diffractometry (XRPD), polarized light microscopy (PLM), Fourier-transform infrared (FTIR) spectroscopy, and modulated differential scanning calorimetry (MDSC). XRPD studies indicate that Mannitol remained amorphous up to 1:5 w/w βLg, Mannitol ratio, whereas PLM showed the presence of crystals of Mannitol in all dried samples except for the 1:1 wt/wt βLg: Mannitoldried sample. FTIR studies indicated that a small proportion of crystalline Mannitol is present along with the amorphous Mannitol in dried samples at lower (less than 1:5 wt/wt) βLg: Mannitol ratios. The Tg of the dried 1:1 wt/wt βLg: Mannitol sample was observed at 33.4°C in MDSC studies, which indicates that at least a part of Mannitol co-exist with protein in a single amorphous phase.[31]

Zhai S *et al.*, (2003)studied to explain the mass transfer processes that influence the rate of primary drying during lyophilization. They showed that the total time required to sublime water from aqueous slurries of glass beads in a conventional laboratory lyophilizer are in reasonable agreement with those times estimated using the values determined by freeze-drying miscroscopy.[32]

Nail.S.L*et al.*, (2002) explained the principles involved, process development methods, advantages, disadvantages and applications of freeze drying

and described about different freeze drying equipments, and pharmaceutical and biological products that can be lyophilized by freeze drying process.[33]

Teagarden DL *et al.,* (2002)studied thatNon-aqueous co-solvent systems have been evaluated for their potential use in the freeze-drying of pharmaceutical products. The advantages of using these non-aqueous solvent systems include increased drug wetting or solubility, increased sublimation rates, increased pre-dried bulk solution or dried product stability, decreased reconstitution time, and enhancement of sterility assurance of the pre-dried bulk solution. Conversely, the potential disadvantages and issues which must be evaluated include the proper safe handling and storage of flammable and/or explosive solvents, the special facilities or equipment which may be required, the control of residual solvent levels, the toxicity of the remaining solvent, qualification of an appropriate GMP purity, the overall cost benefit to use of the solvent, and the potential increased regulatory scrutiny. The co-solvent system that has been most extensively evaluated is the tert-butanol/water combination. The tert- butanol possesses a high vapor pressure, freezes completely in most commercial freeze-dryers, readily sublimes during primary drying, can increase sublimation rates, and has low toxicity. This co-solvent system has been used in the manufacture of a marketed Injectable pharmaceutical product. When using this solvent system, both formulation and process control requires optimization to maximize drying rates and to minimize residual solvent levels at the end of drying. Other co-solvent systems which do not freeze completely in commercial freeze-dryers are more difficult to use and often resulted in unacceptable freeze-dried cakes. Their use appears limited to levels of not more than 10%.[34]

SchoenaMP*et al.*,(1999)studied a lyophilization process model which is adapted to fit experimental data from product vials processed using a development scale dryer. The model is evaluated with regard to how well it is simulated with the primary drying time and temperature conditions for product vials during the primary drying phase of the cycle. The results indicate that the predicted drying time is very close to the actual drying time observed for the product. The simulated product temperature profile is also compared well with the actual product temperature profile.[35]

Liapis *et al.*, (1995) constructed a theory to describe quantitatively the dynamic behavior of the primary and secondary drying stages of the freeze-drying of pharmaceutical crystalline and amorphous solutes. Experimental data for the freeze-drying of cloxacillin monosodium salt and skimmed milk are obtained using a pilot freeze-dryer. The comparison of the theoretical results with the experimental data shows that the agreement between experiment and theory is good.[36]

U.S. Food and Drug Administration (USFDA) has given various guidelines to be followed for the successful manufacturing of parenteral lyophilization product. It explains bulk solution manufacturing, filling, partial stoppering, loading into lyophilizer and freeze drying in chamber and unloading from chamber and sealing and evaluation of finished product.

3. AIM & OBJECTIVE

3.1 AIM

The aim of my present study was to formulate a stable lyophilized formulation of Bendamustine Hydrochloride (100mg/vial) which is therapeutically equivalent to the innovator product, Treanda, manufactured by Cephalon.

3.2 OBJECTIVE

Bendamustine Hydrochloride is an Antineoplastic agent used in the treatment of patients with chronic lymphocytic leukemia (CLL) and with indolent B-cell non-Hodgkin's lymphoma (NHL).It is under the class of alkylating agents.

The purpose of the present study was to formulate a stable lyophilized formulation of the drug Bendamustine Hydrochloride (100mg/vial).

Bendamustine Hydrochloride is unstable in the solution form. So, it cannot be formulated as a liquid dosage form. In order to improve its stability, the drug product should be dried and formulated as a solid product. Even though there are several drying techniques to dry a product, Lyophilization technique was preferred because it involves the drying of the product at low temperature and low pressure. As Bendamustine Hydrochloride is thermo labile in nature and cannot withstand elevated temperatures, this technique is the best choice to increase its stability.

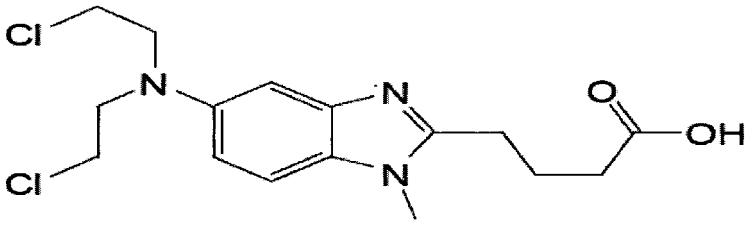
4. PLAN OF WORK

To achieve the ultimate goal of formulating lyophilized product of Bendamustine Hydrochloride, the present work was designed to address the following objectives

- Preformulation studies of the drug
- Formulation of the injectable dosage form
- Development of Lyophilization cycle.
- Compatibility studies with process components
- Lyophilization of the injectable dosage form
- Evaluation of lyophilized product.
- Comparative study with innovator product

5 DRUG AND EXCIPIENT PROFILE[37-39]

Table 3

Name of the Drug Substance	Bendamustine Hydrochloride
Therapeutic Category	Antineoplastic agent
CHEMISTRY	
Nomenclature	4-[5-[Bis(2-chloroethyl)amino]-1-methylbenzimidazol-2-yl]butanoic acid
Molecular formula	$C_{16}H_{21}C_{l2}N_3O_2$
Molecular weight	394.7
Chemical category	Benzimidazole derivative.
Molecular structure	
PHYSICAL PROPERTIES	
Description	White to off white powder
Solubility	Sparingly Soluble in water, soluble in ethanol.
Polymorphism	Exists. Both crystalline and amorphous forms.
Melting point	149-151°C
Dissociation constant(pKa)	4.17
P^H (0.5g of sample in 25ml of carbon dioxide free water)	2.5-3.5
Partition coefficient (log P)	1.10

CHEMICAL PROPERTIES	
Spectral absorbance	Maximum 275 nm
Related substances	Monohydroxy, IPA ester, Dihydroxy and BND-IV impurities.
STABILITY	
Thermal stability	Unstable
Photo stability	Photo stable.
GENERAL	
Handling	Wear suitable protective clothing and gloves. Respiratory protection is required when dusts are generated.
Storage	Stored up to 25°C (77°F) with excursions permitted up to 30°C (86°F).

PHARMACOLOGICAL PROPERTIES

PHARMACOKINETICS

Absorption

Following a single IV dose of Bendamustine hydrochloride C_{max} typically occurred at the end of infusion.

Distribution

Plasma protein binding ranges from 94% to 96%. Steady-state Vd is approximately 25 L.

Metabolism

Metabolized by hydrolysis to compounds with low cytotoxic activity, two active minor metabolites, M3 and M4, are formed via CYP1A2

Elimination

Approximately 90% recovered in excreta, primarily in the feces. Bendamustine clearance in humans is approximately 700 mL/minute. After a single dose of 120 mg/m^{2}bendamustine IV over 1-hour the intermediate $t_{½}$ of the parent compound is approximately 40 minutes. The mean apparent terminal elimination $t_{½}$ of M3 and M4 are approximately 3 hours and 30 minutes respectively. Little or no accumulation in plasma is expected for bendamustine administered on Days 1 and 2 of a 28-day cycle.

Half-life

Intermediate half-life of the parent compound is 40 min; the terminal elimination half-life of M3 and M4 are approximately 3 h and 30 min, respectively. Cl is approximately 700 mL/min.

MECHANISM OF ACTION

Bendamustine is a bifunctionalmechlorethamine derivative containing a purine-like benzimidazole ring. Mechlorethamine and its derivatives form electrophilic alkyl groups. These groups form covalent bonds with electron-rich nucleophilic moieties, resulting in interstrand DNA cross links. The bifunctional covalent linkage can lead to cell death via several pathways. Bendamustine is active against both quiescent and dividing cells. The exact mechanism of action of bendamustine remains unknown.

DOSAGE AND METHOD OF ADMINISTRATION

Dosing Instructions for chronic lymphocytic leukemia (CLL)

The recommended dose is 100 mg/m^2 administered intravenously over 30 minutes on Days 1 and 2 of a 28-day cycle, up to 6 cycles.

Dosing Instructions for B-cell non-Hodgkin's lymphoma (NHL)

Recommended Dosage

The recommended dose is 120 mg/m2 administered intravenously over 60 minutes on Days 1 and 2 of a 21-day cycle, up to 8 cycles.

WARNINGS AND PRECAUTIONS

Renal Function

Use with caution in patients with mild or moderate renal impairment. Do not use in patients with CrCl less than 40 mL/min**.**

Hepatic Function

Use with caution in patients with mild hepatic impairment. Avoid use in patients with moderate to severe hepatic impairment**.**

Anaphylaxis

Anaphylaxis and anaphylactoid reactions have been reported, especially in the second and subsequent cycles of therapy**.**

Infections

Infections, including pneumonia and sepsis, have been reported. Patients experiencing myelosuppression after treatment are more susceptible to infections.

Infusion reactions

Reported frequently in clinical trials. Symptoms include chills, fever and rash.

Malignancies

Premalignant and malignant diseases, including acute myeloid leukemia and bronchial carcinoma, myelodysplastic syndrome, and myeloproliferative disorders, have been reported**.**

Skin reactions

Have been reported and may include bullous exanthema, toxic skin reactions, and rash**.**

DRUG INTERACTIONS

Inducers of CYP1A2 (e.g., omeprazole, smoking)

May decrease bendamustine plasma concentrations and increase levels of its active metabolites. Co-administer with caution.

Inhibitors of CYP1A2 (e.g., ciprofloxacin, fluvoxamine)

May increase bendamustine plasma concentrations and decrease levels of its active metabolites. Co-administer with caution.

UNDESIRABLE EFFECTS

Cardiovascular

Tachycardia, Hypotension, Cardiac failure

CNS (central nervous system)

Fatigue, headache, dizziness, insomnia, asthenia, anxiety, depression

Dermatological

Rash, Pruritus , dry skin, hyperhidrosis, night sweats ,skin necrosis, skin reactions, including Stevens-Johnson syndrome and TEN (post marketing).

GI (gastro intestinal)

Nausea ,vomiting , diarrhoea , constipation, Stomatitis , abdominal pain, decreased appetite , dyspepsia, gastro esophageal reflux disease, dry mouth ,oral candidiasis, abdominal distension, upper abdominal pain.

Local

1) Infusion-site pain, catheter-site pain, infusion reactions, injection-site irritation, pain and swelling

Metabolic-Nutritional

Anorexia, decreased weight, dehydration, peripheral edema, hypokalemia, hyperuricemia, hyperglycemia, hypocalcemia, hyponatremia

Musculoskeletal

Back pain, Arthalgia, bone pain, pain in extremities

Respiratory

Cough dysponea, upper respiratory tract infection, sinusitis, pneumonia, and wheezing, pulmonary fibrosis.

Miscellaneous

Chills, herpes zoster, chest pain, febrile neutropenia, infection, herpes simplex, myelodysplastic syndrome, sepsis, tumor lysis syndrome

OVERDOSE

Symptoms

ECG changes, including QT prolongation, sinus tachycardia, ST- and T-wave deviations, and left anterior fascicular block.

No specific antidote for TREANDA overdose is known. Management of over dosage should include general supportive measures, including monitoring of hematologic parameters and ECGs.

5.2 EXCIPIENTS PROFILE [40]

Substances, other than the active ingredient, which have been appropriately evaluated for safety and are included in a drug delivery system to provide support.

The excipients used must have following characteristics-

1. They must be stable both physically, chemically and must be biologically inactive.
2. It must be free from microbial contamination
3. Excipients used in formulation must be accepted by regulatory agencies and should meet the entire current regulatory requirement.

The literature review of the innovator drug provided the qualitative and quantitative composition of the product. So, the same excipients (Mannitol) and solvents (Tertiary butyl alcohol, Water for Injection) which were present in the innovator product were selected for the present study.

5.2.1 Mannitol

a) Nonproprietary Names

BP: Mannitol

IP: D-Mannitol

PhEur: Mannitol

USP: Mannitol

b) Synonyms

Cordycepic acid, C*PharmMannidex, E421, Emprove; mannasugar, D-mannite, mannite, mannitolum, Mannogem, Pearlitol

c) Chemical Name and CAS Registry Number

D-Mannitol [69-65-8]

d) Empirical Formula

$C_6 H_{14}O_6$

e) Molecular Weight

182.17

f) Functional Categories

Diluents, plasticizer, sweetening agent, tablet and capsule diluents, therapeutic agent, tonicity agent

g) Applications in Pharmaceutical Formulation or Technology

Mannitol is widely used as a diluent (10–90% w/w) in tablet formulations, since it is not hygroscopic and may thus be used with moisture sensitive active ingredients. It may be used in direct compression tablet applications, for which the granular and spray dried forms are available, or in wet granulations. Granulations containing Mannitol have the advantage of being dried easily.

It is also used as an excipient in the manufacture of chewable tablet formulations because of its negative heat of solution, sweetness, and 'mouth feel' and also used as a diluent in rapidly dispersing oral dosage forms

In lyophilized preparations, Mannitol (20–90% w/w) has been included as a carrier to produce a stiff, homogeneous cake that improves the appearance of the lyophilized plug in a vial. A pyrogen-free form is available specifically for this use.

It has also been used to prevent thickening in aqueous antacid suspensions of aluminum hydroxide (<7% w/v), and has been suggested as a plasticizer in soft-gelatin capsules, as a component of sustained-release tablet formulations and as a carrier in dry powder inhalers.

Therapeutically, Mannitol administered parenterally is used as an osmotic diuretic, as a diagnostic agent for kidney function, as an adjunct in the treatment of acute renal failure, and as an agent to reduce intracranial pressure, treat cerebral edema, and reduce intraocular pressure.

h) Description

It occurs as a white, odorless, crystalline powder, or free flowing granules. It has a sweet taste and imparts a cooling sensation in the mouth. Microscopically, it appears as orthorhombic needles when crystallized from alcohol.

i) Incompatibilities

Mannitol solutions, 20% w/v or stronger, may be salted out by potassium chloride or sodium chloride. Precipitation has been reported to occur when a 25% w/v solution was allowed to contact plastic.

It is incompatible with xylitol infusion and may form complexes with some metals such as aluminum, copper, and iron. Reducing sugar impurities in it have been implicated in the oxidative degradation of a peptide in a lyophilized formation. It was found to reduce the oral bioavailability of cimetidine compared to sucrose.

j) Stability and Storage Conditions

It is stable in the dry state and in aqueous solutions. In solution, it is not attacked by cold, dilute acids or alkalis or by atmospheric oxygen in the absence of catalysts. It does not undergo Mallard reactions.

The bulk material should be stored in a well-closed container in a cool, dry place.

5.2.2 TBA (Tertiary Butyl Alcohol)

a) Empirical Formula : $C_4 H_{10}O$

b) CAS no: 75-65-0

c) Molecular Weight: 74.12

d) Physical State and Color: Colorless liquid which forms rhombic-like crystals

e) Melting Point: 25.6 ℃- 25.7 ℃

f) **Boiling Point**: 82.41 ℃

g) Specific Gravity: 0.78086

h) Vapor Pressure: 30.6 mm Hg @ 20 ℃; 42 mm Hg @ 25 ℃

i) Solubility: Soluble in water.

j) Flash point: 110 ℃

k) Explosion limits: 2.4 - 8%

l) Stability

Stable. Very flammable Incompatible with strong oxidizing agents, copper, alloys, alkali metals, aluminium.

m) Toxicology

Harmful if inhaled. Skin and respiratory irritant, Severe eye irritant, Typical TLV (Threshold Limit Value) /TWA (time weighted average) - 100 ppm. Typical STEL (short term exposure limit) -150 ppm

n) Residual solvent limit in lyophilized product

NMT 3000 ppm

6.MATERIALS AND METHODS

6.1 LIST OF METERIALS

Table 4List of Raw Materials and Their Source

S.No	INGREDIENT	VENDOR
1	Bendamustine Hydrochloride	NatcoPharma
2	Mannitol 25	Roquette
3	Tertiary butyl Alcohol	Merck
4	Water For Injection (WFI)	NatcoPharma

Table 5 List of Packaging Materials and Their Source

S.No	PRIMARY PACKING MATERIAL	GRADE	VENDOR
1	50ml Glass Vials – USP Type – I	USP	MatriMirra
2	20 mm Double slotted Bromobutylrubber stoppers	USP/EP	West Pharmaceuticals
3	Aluminium flip off seals	IH	HBR Packaging India

Table 6List ofEquipments

S.No	NAME OF THE EQUIPMENT	MANUFACTURER	USE OF EQUIPMENT
1	Lyophilizer	LSI(lyophilization systems India pvt ltd) and Lyolab	To perform the process of Freeze drying
2	Weighing balance	Mettler Toledo	To weigh the raw materials and finished product
3	pH meter	Lab India PICO+	To find out the pH of the product before and after Lyophilization
4	DO meter	HACH Ultra	To find the dissolved oxygen content of the solution
5	Filtration unit	Millipore	To clarify the drug solution
6	Osmometer	Advanced instruments, INC 3250	To determine the osmolality of solution.
7	HPLC	Waters 2487	To know the assay and related substances of the drug.
8	KF titrator	MettlerToledo,DL50 GRAPHIX	To determine the water content of lyophilized drug
9	Stability chambers	Thermo-lab	To conduct the stability studies of the drug product

Table 7List of Chemicals and Reagents used for HPLC

S.No	Reagents	Grade	Manufacturer
1	Trifluoroacetic acid	HPLC grade	Merck
2	Acetonitrile	HPLC grade	Merck
3	Methanol	HPLC grade	Merck
4	Potassium dihydrogenorthophosphate	GR-grade	Merck
5	Triethyl amine	HPLC grade	Merck
6	Methanol	HPLC grade	Merck
7	Isopropyl Alcohol	HPLC grade	Merck

6.2 PREFORMULATION STUDIES

Preformulation studies are performed to investigate the physical and chemical properties of a drug substance alone and also when combined with other substances such as excipients. It is the first step in the rational development of dosage forms.

The overall objective of performing preformulation testing is to generate information that will be helpful in developing a stable and bioavailable dosage form when combined with excipients.

The use of Preformulation parameters maximizes the chances in formulating an acceptable, safe, efficacious and stable product and at same time provides the basis for optimization of the drug product quality.

6.2.1 Preparation of Standard Graph

Bendamustine Hydrochloride (20.0mg) working standard was accurately weighed and transferred into 200 mL amber colored volumetric flask. To this 60 ml of methanol was added, it was dissolved and the volume was made up to the mark with methanol. Followed by serial dilution method, the concentrations of 5, 10, 20,

40, 80,160(mcg/ml) of Bendamustine Hydrochloride was analyzed by HPLC at wave length of 275nm and the peak areas were noted. A standard graph of the peak areas versus the concentration of the drug was plotted.

6.2.2 Organoleptic Properties

The color, odour and taste of the drug were recorded using descriptive terminology.

6.2.3 Saturation Solubility Studies

a) In water

It was determined by adding excess Bendamustine Hydrochloride in a fixed volume (10ml) of water. It was kept under shaking for 24hrs on a shaker. It is further filtered and analyzed using HPLC at 275nm.

b) In water and alcohol

The solubility of bendamustine hydrochloride in water and with varying amounts of alcohols commonly used in lyophilization.

E.g.: methanol,ethanol,propanol,butanol, and TBA (territory butyl alcohol) was determine by visual inspection.

Amount of bendamustine hydrochloride at 15 mg/ml, combined with mannitol at 25.5 mg/ml were prepared in 10 ml of the individual alcohol solutions at room temperature. Sample ware then refrigerated at 5^{o} C and inspected after 0, 3,6,24 hrs for particulates and/or precipitates

6.2.4 Solution Stability Studies

The stability studies in TBA were carried out as follows:

WFI (100ml) was taken in a beaker. To this required quantity of TBA, Mannitol&Bendamustine Hydrochloride was added and is dissolved by stirring. The

volume is made up to 200ml with WFI. This preparation was divided into 8 lots from which 4 lots were stored at 25^0C, while remaining 4 lots were stored at 2 - 8^0C for 24 hours. Further analysis was performed after each interval i.e. 3,6,9,12,24 hrs.

Table 8 TBA stability studies at different level of temperatures (25^0C and at 2-8 ^{0}C)

S.No	Ingredient	TBA 10%		TBA 20%		TBA 30%		TBA 40%	
		Qt/ml	Qt/200ml	Qt/ml	Qt/200ml	Qt/ml	Qt/200ml	Qt/ml	Qt/200ml
1	B.H	5mg	1gm	5mg	1gm	5mg	1gm	5mg	1gm
2	Mannitol	6mg	1.2gm	6mg	1.2gm	6mg	1.2gm	6mg	1.2gm
3	TBA	0.1ml	20ml	0.2ml	40ml	0.3ml	60ml	0.4ml	80ml
4	WFI	q.s	q.s	q.s	q.s	q.s	q.s	q.s	q.s

B.H=Bendamustine Hydrochloride, WFI=Water for injection, TBA = tertiary butyl alcohol, q.s=quantity sufficient

6.3 FORMULATIONOFBENDAMUSTINE HYDROCHLORIDEINJECTION

Table 9 manufacturing formula

S.No	Ingredients	Category
1	Bendamustinehydrochloride	Active ingredient
2	Mannitol	Lyophilization aid
3	Tertiary butyl alcohol	Solvent
4	Water for injection (WFI)	Diluent

6.4 FORMULATION OF BENDAMUSTINE HYDROCHLORIDE

Different formulations of pre-lyophilized bendamustine hydrochloride injection were prepared with different solvent concentration of TBA and WFI. The formulation were shown in table 11.the cycle development was changed and optimized at each step for freezing (fast Vsslow), primary drying (both temperature and pressure) and secondary drying.

Table 10Formulation of Bendamustine Hydrochloride

S.no	Ingredients	FI	FII	FIII	FIV
1	B.H (gm)	1	1	1	1
2	Mannitol (gm)	1.2	1.2	1.2	1.2
3	TBA (ml)	-	40	60	80
4	WFI	200	160	140	120

B.H=Bendamustine Hydrochloride, WFI=Water for injection, TBA = Tertiary Butyl Alcohol

Table 11 formulafor single vial

S.No	Ingredients	Qt/ml	Qt/vial (20 ml)
1	Bendamustine hydrochloride	5mg	100 mg
2	Mannitol	6 mg	120 mg
3	Tertiary butyl alcohol	0.3 ml	6 ml
4	Water for injection	q.s	q.s

Manufacturing procedure

1. About 50% of WFI (water for injection) was taken in a beaker and cooled at 25^0 C. To this dispensed quantity of TBA (tertiary butyl alcohol) was added and mixed well until homogeneous solution is obtained. This solution was cooled to 2-8^0C .The pH of the solution was found to be 6.8.
2. Weighed quantity of Mannitol was added to the above solution and dissolved by stirring at a speed of 200 RPM under 2-8 ^{0}Ccondition until clear solution was observed.
3. Weighed quantity of Bendamustine Hydrochloride was added and dissolved by stirring for 30 minutes at a speed of 200 RPM under 2-8^0C condition. The pH of the solution was found to be 3.1

4. The solution was then made up to the volume 200 ml with WFI under 2-8^{o}C condition. The final pH of the solution was found to be 3.1
5. The final solution was filtered using 0.22-µm PVDF (poly vinyl disposable filter).
6. The solution was filled into 50 ml of amber colored USP-Type-1 vial with target fill volume of 20 ml with pre and post purging of Nitrogen half stoppered, the vials with 20mm Lyophilized stoppers
7. Filled vials are loaded into the lyophilizer and lyophilized the vials as per cycle conditions mentioned in Table 15 (Set the shelf temperature prior to cycle staring to 5^{o}c)
8. The samples are collected after the completion of the cycle. The samples are withdrawn under the nitrogen atmospheric conditions after the atmospheric pressure is reached, stoppered and sealed. The vials are unloaded from the lyophilizer and sealed using flip off aluminum seals &Lyophilized vials are stored at room temperature.

6.5 COMPATIBILITY STUDIES WITH PROCESS COMPONENTS

Definition: Compatibility is a measurement of how stable a substance is when it is in contact with another material. If there is no change in the physical and chemical properties of the substance when it is in contact with other materials, then it is considered as compatible. If changes occur, then it is considered as incompatible.

Types of Compatibility Studies:

1. Filter compatibility
2. Silicone Tubing compatibility
3. SS Vessel compatibility

6.5.1 Filter Compatibility Study

- Filter material must be compatible with the chemical nature of the sample stream under the conditions at which the filtration will be performed. This will minimize the risk of structural failure during filtration.

- The purpose of this study was to determine the effect of stability on prolonged exposure of filter membrane to drug product.

a) Study Design:

This study can be done in two ways.

a) Static Soak Method*:*

In this study, the test filter shall be soaked in the drug product solution for longer period.

b) Dynamic Filtration*:*

In this study, the liquid is continuously circulated through the filter membrane repeatedly.

Of these both methods, static soak method was used for the present study.

6.5.1.1 Experimental procedure

- The test filter (PVDF filter) shall be subjected to autoclaving and then use it for the compatibility studies.

- Place the filter membrane in drug product solution and keep aside at 2-8ºC.

- At regular time intervals (0, 2, 4, 6 hours) withdraw the required amount of sample and perform analysis of it.

6.5.2 Silicone Tubing Compatibility Study

- Silicone tubing's are commonly used as processing aid during filtration and filling operation for transfer of liquid in pharmaceutical manufacturing. They are known to adsorb or interact with product and may lead to instability of product.
- The purpose of this study was to determine the effect of silicone tubing exposure on drug product characteristics.
- In the present study Pharma Pure silicon tubing was used.

a) **Study Design:** This study shall be planned in two ways.

b) **Static Condition:** In this case the liquid is hold in the tube for longer period.

c) **Dynamic Condition***:* In this case the liquid is continuously passed from the tubing at a constant flow rate for required time period.

Of these both conditions, static method was used for the present study.

6.5.2.1 Experimental procedure

- Collect the required length of tubes and check the tubes physically for any damage
- Wash the tubes using potable water followed by WFI and wrap in autoclavable pouch and sterilize at 121°C for 30 minutes.
- Open the tube under laminar air flow and check for any damage after sterilization and then fill the tube with drug product solution.
- Close the both ends of tube and kept at 2-8°C.
- At regular time intervals (0, 2, 4, 6 hours) withdraw the required amount of sample and perform analysis of it.

6.5.3 SS Vessel Compatibility Study

- SS vessels are commonly used in fabrication, filtration and filling of drug products. During this hold period the drug product may interact with SS 316 vessel and this may lead to incompatibility.

- The compatibility study with SS vessel is required to establish holding period.

6.5.3.1 Experimental procedure

- Collect the SS 316 vessel. Wash it and sterilize at 121°C for 30 min.

- Fill the drug solution into the SS vessel after its filtration and then close the container.

- At regular time intervals (0, 2, 4, 6 hours) withdraw the required amount of sample and perform analysis of it.

6.6 LYOPHILISATION CYCLE DEVOLOPMENT

Table 12 Different lyophilization conditions with varying temperature and duration of cycle

S.no		F 1			F 2			F 3			F 4		
		T 1	T 2	T 3	T 1	T 2	T 3	T 1	T 2	T 3	T 1	T 2	T 3
1	FREEZING												
	Rate of freezing	Fast	Fast	Fast	Fast	Slow	Slow	Fast	Slow	Slow	Fast	Slow	Slow
	Duration (hrs)	4.5	6.5	7.58	7.58	23.25	18.8	7.58	23.25	18.8	7.58	23.3	18.8
	Final freezing temperature (˚C)	-40	-45	-45	-40	-45	-45	-40	-45	-45	-40	-45	-45
	Annealing step	No	No	No	No	Yes	Yes	No	Yes	Yes	No	Yes	Yes
2	PRIMARY DRYING												
	Duration (hrs)	19	27	43.5	29	32.16	53	29	32.16	53	29	35.6	53
	Final shelf temperature (˚C)	40	40	40	30	35	35	30	35	35	30	35	35
3	SECONDARY DRYING												
	Duration (hrs)	2	8.5	8	11	14.1	14.1	11	14.1	14.1	11	16	14.1
	Final shelf temperature(°C)	40	40	40	35	35	40	35	35	40	35	35	40
	Entire cycle duration (hrs)	25.5	42	59.08	47.58	69.5	85.9	47.5	69.5	85.9	47.5	69.5	85.9

6.7 EVALUATION OF LYOPHILIZED PRODUCT

The lyophilized product was evaluated for the following formulation characteristics:

- Description
- Reconstitution time
- Clarity of reconstituted solution.
- pH after reconstitution
- Water content
- Assay
- Related substances
- Diluent compatibility studies.
- Stability studies as per ICH guidelines
- Photo stability studies.

6.7.1 Description

It involves the physical examination of the sample after lyophilization.

6.7.2 Reconstitution Time

Vial was reconstituted (**100mg – 20 ml)** with water for Injection.

Water for injection was slowly injected directing the syringe tip onto the wall of the vial.

The vial was gently shaken by tilting the vial upside down until the cake dissolves and the Timewas noted till it completely dissolves and this was called as Reconstitution time.

The final solution was preserved for the test clarity and color of the solution.

6.7.3 Clarity of Reconstituted Solution

The above preserved solution in the test for “Reconstitution time” was transferred into a clear test tube and the clarity and color of the solution was observed.

The solution should be clear colorless leaving no visible residue as undissolved matter.

6.7.4 pHafter Reconstitution

The vials were reconstituted with carbon-dioxide free water and thenthe pH of the solution was measured using a suitable calibrated pH meter.

6.7.5 Water Content

The amount of water present in the lyophilized drug was determined using Karl Fisher Titrimetry method.

Procedure

Methanol (20ml) was taken into the titration vessel and titrated the amperometric end point with Karl Fischer Reagent. To this 0.1 gm of the sample was added, it is stirred for 1 minute and again titrated to the amperometric end point with Karl Fischer Reagent.

$$\frac{(\mathbf{T.V \times K.F.\ FACTOR \times 100})}{\mathbf{Wtofsample \times 100}}$$

***T.V=Titrevalue,K.F=Karl fischer**

6.7.6 Assay

Assay was done to find and measure the amount of drug in the final product.

In the present study, HPLC method was used to determine the assay.

Preparation of Buffer

Potassium dihydrogen orthophosphate (6.8) was dissolved in 1000 mL of purified water, the pH was adjusted to 7.0 ±0.1 with Triethylamine. The solution was mixed, filtered through 0.45μmmembrane filter and degas.

Preparation of Mobile phase

The Buffer and Methanol mixture was prepared in the ratio of 370:630 v/v, filtered through 0.45μm membrane filter and degas.

Diluent

Methanol was used as diluent.

Table 13Chromatographic conditions

Column	Develosil, ODS UG-5, (150 × 4.6mm)
Flow rate	0.8 mL / minute
Wavelength	UV-275 nm
Column temperature	30°C
Sample temperature	5°C
Injection Volume	10μL
Run time	12 minutes

Preparation of sample solution

5 vials were taken and they are reconstituted with 40 mL of diluent, without any loss of solution and these are transferred into 500 mL amber colored volumetric flask.The vials were washed with 10 mL of diluents for 2 -3 times and the volume was made up to 500 mL with diluents and mixed well.

2.0 mL of the above solution was transferred into 100 mL amber colored volumetric flask, and the volume was made up with the diluents.

Procedure

10μL of diluent as blank, standard preparation, sample preparations are separately injected into the chromatograph, the peak area responses are measured from the obtained chromatograms. The % content of Bendamustine Hydrochloride in the portion of Bendamustine Hydrochloride injection was calculated by using the formula

% Content of Bendamustine (mg/vial)

$$\frac{TA}{SA} \times \frac{SW}{200} \times \frac{5}{25} \times \frac{500}{5} \times \frac{100}{2} \times \frac{P}{100} \times \frac{100}{LA}$$

Where,

TA: peak area response due to Bendamustine Hydrochloride from sample preparation.

SA: peak area response due to Bendamustine Hydrochloride from standard preparation.

SW: Weight of Bendamustine Hydrochloride working standard taken in mg.

P: purity of Bendamustine Hydrochloride working standard taken on as is basis.

LA: Label Amount in mg.

6.7.7 Related Substances

Related substances are the unwanted substances or the degradants which may be present in the final product along with the drug substance. Thus they form the impurity of the drug product. So these are to be found out and the total impurity has to be calculated.

HPLC method was used to determine the amount of related substances.

a) Preparation of Mobile phase -A

1.0 mL of TrifluoroaceticAcidwas mixed in 1000mL of water, filtered through 0.45 μm membrane filter and degas.

b) Preparation of Mobile phase – B

1.0 mL of Trifluoroacetic Acid was mixed in 500mL of water, to this 500mL Acetonitrile was added, filtered through 0.45 μm membrane filter and degas.

c) Diluent

Methanol was used as diluent.

Table 14Chromatographic conditions

Column	Develosil, UG-5, C-18 (250 × 4.6mm)
Flow rate	0.8 mL / minute
Wavelength	UV-275 nm
Column temperature	30°C
Sample temperature	5°C
Injection Volume	15μL
Run time	15 minutes

Table 15Gradient programming

Time(min)	Flow(mL/min)	Mobile phase-A (% v/v)	Mobile phase-B (% v/v)
0	0.8	75	25
2	0.8	75	25
10	0.8	60	40
18	0.8	60	40
25	0.8	20	80
35	0.8	20	80
39	0.8	15	85

PREPARATION OF SOLUTIONS

a) Preparation of standard solution

Accurately weigh and transfer about 20.0 mg of Bendamustine Hydrochloride working standard into a 200-mL amber colored volumetric flask. Add about 120 mL of diluents and sonicate to dissolve then make up to the volume with diluents and mix well.

Transfer 1.0 ml of the above solution into a 200 mL amber colored volumetric flask, dilute to volume with diluents

b) Peak identification solution preparation

Accurately weigh and transfer about each 5.0 mg of Dihyroxy Impurity, Monochloro impurity, Monohydroxy Impurity, BND-VI Impurity, IPA Ester Impurity and Bendamustine Hydrochloride working standard into 100 mL amber colored volumetric flask and add 60mL diluent, sonicate to dissolve and dilute to volume with diluents.

Transfer 2.0 mL of the above solution into a100 mL amber colored volumetric flask and dilute to volume with diluent.

c) Preparation of sample solution (For Bendamustine Hydrochloride Injection 100mg/vial)

Take 2 vials and reconstitute each vial with 10mL of diluents, dissolve and transfer the contents into 200 mL amber colored volumetric flask without any loss of solution. Wash the vials with 10 mL of diluents for 2-3 times and make upto the volume to 200 mL with diluents, shake and mix well.

Transfer 10.0 mL of the above solution into a 20 mL volumetric flask, dilute to volume with diluents

d) Procedure

15µL of diluents as blank, placebo, peak identification solution, standard preparation and sample preparations are injected into the chromatograph, the peak area responses are measured from the obtained chromatograms. The % content of Bendamustine Hydrochloride in the portion of Bendamustine Hydrochloride injection was calculated by using formula.

Table 16 RRT and RRF of different components

Component Name	Relative Retention Time (RRT)	Relative Retention Factor (RRF)
Bendamustine	About 1.00	1.00
Dihydroxy Impurity	About 0.20	0.28
Monochloro Impurity	About 0.59	0.53
Monohydroxy Impurity	About 0.63	0.88
BND-VI impurity	About 0.46	0.74
IPA Ester Impurity	About 1.41	0.81

Calculations (For Bendamustine Hydrochloride Injection 100mg/vial)% of Known impurities

$$\frac{IA}{SA} \times \frac{WS}{200} \times \frac{1}{200} \times \frac{200}{2} \times \frac{20}{10} \times \frac{P}{100} \times \frac{1}{LA} \times \frac{100}{RRF}$$

% **of Unknown impurities**

$$\frac{IA}{SA} \times \frac{WS}{200} \times \frac{1}{200} \times \frac{200}{2} \times \frac{20}{10} \times \frac{P}{100} \times \frac{1}{LA} \times 100$$

Total Impurities

Sum of Known impurities + Unknown impurities

UA : Peak area response due to Un-Known impurity from sample preparation.

SA : Peak area response due to Bendamustine Hydrochloride from standard preparation.

WS : Weight of Bendamustine Hydrochloride working standard taken in mg.

P : purity of Bendamustine Hydrochloride working standard taken on as is basis.

LA : Label Amount of Bendamustine Hydrochloride.

RRF : Relative Response Factor of respective known impurity.

IA : Peak area response due to Known impurity from sample preparation

6.8 DILUENT COMPATIBILITY STUDIES

OBJECTIVE

This study aims to establish the compatibility of Bendamustine Hydrochloride lyophilized product with diluents such as 0.9% Sodium Chloride Injection, USP or 2.5% Dextrose injection/0.45 Sodium chloride injection, USP which are recommended for dilution in the package insert of innovator for the recommended duration.

a) Procedure:

The powder was immediately reconstituted after opening of the vial (100mg) with 40 ml of water for injection (WFI). The reconstituted concentrate is diluted immediately with 0.9% NaCl solution (500ml).

After reconstitution and dilution chemical and physical stability has been demonstrated for 3.5hrs at 25^0C and for 48 hrs at 2-8^0C in polyethylene bags.

The various test parameters that are evaluated include:

- Description
- pH
- Assay
- Related substances
- Osmolality

Table 17 Acceptance criteria

S.No	TEST PARAMETER	LIMITS
1	Description	Clear, Colorless solution.
2	PH	Between 2.5-3.5
3	Assay (%)	90 – 110
4	Related substances (%)	NMT 3
5	Osmolality (m.osm)	290- 310

B) Osmolality

The powder was immediately reconstituted after opening of the vial (100mg) with 40 ml of water for injection (WFI). The reconstituted concentrate is diluted immediately with 0.9% NaCl solution (500ml). From this 0.2ml of solution is taken in holder and kept in osmometer and freezed for 90 sec the reading is noted.

6.9 INNOVATOR DETAILS

The innovator drug product information can be collected from various sources such as:

(i) USFDA, EMEA, WHO, etc. – for the composition and primary package of innovator product.

(ii) Patent search – for existence or expiration of the patents.

(iii) IIG – for the excipients information.

(iv) Publicly available information (PIL, Product Monographs, Label information, Consumer information, etc.)

(v) Pharmacopoeias – United States Pharmacopoeia-National Formulary (USP-NF), British Pharmacopoeia (BP), European Pharmacopoeia, etc.

(vi) Martindale, Merck index, PDR, Handbook of the excipients and electronic data base (articles and publications) – for the other required information like solubility data, stability data, pH solubility & stability data, compatibility data, etc.

Marketed brand of bendamustine hydrochloride

Figure 7 Innovator product "TREANDA"

Label contains

Vial with 220 mg of powder for infusion solution containing 100 mg of Bendamustine Hydrochloride, main inactive ingredient is Mannitol

Table 18

Name of the product	Treanda
Manufacturer name	Cephalon
Color	White to off white
Description	Lyophilized cake
Package	50ml amber colored USP-Type-1 vial

6.10 STABILITY STUDIES AS PER ICH GUIDELINES[41]

Stability is defined as the capacity of drug product or substance to remain within established specifications to maintain its identity, strength, quality and purity throughout the retest or expiration dating periods.

Stability studies provide an evidence on how the quality of the drug product or substance varies with time under the influence of a variety of environmental factors such as

- Temperature
- Humidity
- Light

These studies establish the

- Re-test period of the drug product
- Shelf life for the drug product
- Recommended storage conditions

In any rationale design and evaluation of dosage forms for drugs, the stability of the active component will be major criteria in determining their acceptance or rejection. So, in the

Present study, the stability of the drug product is assessed by exposing the product to various temperatures and humidity conditions.

a) **Accelerated study**: The product was subjected to accelerated stability studies at 40°C±2°C/75% ±5% RH for 6 months. The frequency of testing at the Accelerated study condition includes 0, 1, 2, 3, 6 months.

b) **Long term study**: The product was subjected to long term studies at 25°C±2°C/60% ±5%RH for 12 months. The frequency of testing at the long term storage condition is for every 3 months over the first year, every 6 months over the second year. Thesampling withdrawn schedule was illustrated in the table below.

Table 19 stability sampling withdrawal schedule

S.NO.	STORAGE CONDITION	TEST PERIOD
1	40°C±2°C/75% ±5% RH	1 month 2 month 3 month
2	25°C±2°C/60% ±5% RH	3 month

Table 20 shelf life specifications for compliance during stability

TESTS	SPECIFICATIONS
Description	White to Off-white lyophilized cake or powder in a tubular50ml clear USP Type - I glass vial sealed with bromobutyl double slotted rubber stopper and aluminum flip-off seal.
pH after reconstitution	2.50 to 3.50
Reconstitution time	NMT 5
Clarity of reconstituted solution	The solid dissolves completely leaving no visible residue as undissolved matter. Reconstituted solution should not be significantly less clear than an equal volume of the diluent
Assay (%)	90-110
Related substances (%)	NMT 3
Osmolality (m.osm)	290 to 310
Water content by KF (%w/w)	NMT 3

6.11 PHOTOSTABILITY STUDY[42]

OBJECTIVE

- This study aims to evaluate the stability of the drug product when exposed to light.
- The intrinsic photostability characteristics of the drug product should be evaluated to demonstrate that, as appropriate, light exposure does not result in an unacceptable change.

STUDY PLAN

Instructions

- The temperature of the photo stability chamber should be maintained at 25°C ± 2°C during the study.

- Vials shall be delabelled for the study. Though the immediate and marketing pack includes the label, in the current study, labels are being removed to study the photo stability characteristics of the product under maximum light stress.

- Samples should be placed horizontally or transversely with respect to the light source, whichever provides maximum light exposure to the samples.

- Check and ensure that photo stability chamber is duly calibrated before use.

Table 21various samples for photo stability studies

SAMPLE	DESCRIPTION	NUMBER OF VIALS
Sample 1 (Product in immediate pack)	Delabelled product vials	20 vials
Sample 2 (Product in marketing pack)	Delabelled product vials placed in cartons	20 vials
Temperature/Dark Control Sample	Delabelled product vials wrapped with Aluminium foil	20 vials

Sample 1:

Collect specified number of vials and place them horizontal to the light source.

Sample 2:

Collect the specified number of vials and place them in opaque white cardboard cartons. The cartons shall be placed in a manner such that the vials placed inside remain in a horizontalcondition. Though no light exposure is expected in these samples, the placement of samples is simulated as closely as possible to the "Sample 1" vials.

Temperature/Dark Control Sample:

Collect the specified number of vials and wrap them thoroughly with aluminium foil leaving no exposed surfaces. Place these vials alongside the "Sample 1" vials in horizontal condition simulating the placement of the "Sample 1" vials. The dark control samples shall be evaluated to understand the contribution of thermally induced changes in the "Sample 1" and "Sample 2" product vials

PROCEDURE

- All the samples shall be exposed to light providing an overall illumination of not less than 1.2 million lux hours and an integrated near ultraviolet energy of not less than 200 watt hours/square meter.

- At the end of the exposure period, the product samples should be examined for the parameters listed below. The analysis of "Sample 1", "Sample 2" and the temperature or dark control samples shall be performed concomitantly.

The various test parameters which were evaluated during this study included:

Table 22 Acceptance criteria

TESTS	SPECIFICATIONS
Description	White to off-white lyophilized cake or powder.
pH after reconstitution	2.50 to 3.50
Reconstitution time	NMT 5 min
Clarity of reconstituted solution	The solid dissolves completely leaving no visible residue as undissolved matter. Reconstituted solution should not be significantly less clear than an equal volume of the diluent
Assay (%)	90-110
Related substances (%)	NMT 3
Water content by KF (%w/w)	NMT3

7. RESULTS

7.1 PREFORMULATION STUDYS

7.1.1 Standard Graph of Bendamustine Hydrochloride

The concentrations of Bendamustine Hydrochloride and the corresponding peak area values are shown in the Table 23 .The plot of concentration versus peak area is shown in the Figure 9.

Table 23Standard graph data of Bendamustine Hydrochloride

S. No.	Concentration(mcg/ml)	Peak Area at 275 nm
1	0	0
2	5	243310
3	10	546620
4	20	1093240
5	40	2286480
6	80	4172960
7	160	8445920

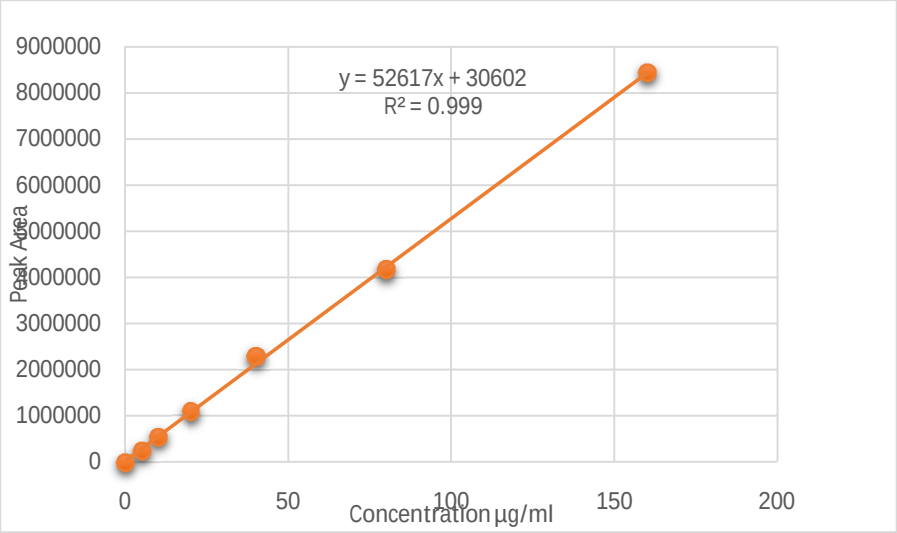


Figure 9Standard graph of Bendamustine Hydrochloride

7.1.2 Description

The received sample of API after visual observation it was found to show the white to off-white color

7.1.3 aturation Solubility Studies

a) In Water

The solubility of Bendamustine hydrochloride was found to be 60mg/ml

b) In Water and Alcohol

Table 24

S.No	Substance	0hrs	3 hrs	6 hrs	24 hrs
1	Water				
2	Methanol (v/v)				
	5%	CCS	CCS	PPT	PPT
	10%	CCS	CCS	PPT	PPT
	20%	CCS	CCS	CCS	PPT
	30%	CCS	CCS	CCS	CCS
3	Ethanol (v/v)				
	5%	CCS	CCS	PPT	PPT
	10%	CCS	CCS	CCS	PPT
	20%	CCS	CCS	CCS	CCS
	30%	CCS	CCS	CCS	CCS
4	n-propanol(v/v)				
	5%	CCS	CCS	CCS	PPT
	10%	CCS	CCS	CCS	CCS
	20%	CCS	CCS	CCS	CCS
	30%	CCS	CCS	CCS	CCS
5	Iso-propanol (v/v)				
	5%	CCS	PPT	PPT	PPT
	10%	CCS	CCS	CCS	CCS
	20%	CCS	CCS	CCS	CCS
	30%	CCS	CCS	CCS	CCS
6	n-butanol (v/v)				
	5%	CCS	CCS	CCS	CCS
	10%	CCS	CCS	CCS	CCS
	20%	2-layers	2-layers	2-layers	2-layers
	30%	2-layers	2-layers	2-layers	2-layers
7	TBA(v/v)				
	5%	CCS	CCS	CCS	PPT
	10%	CCS	CCS	CCS	PPT
	20%	CCS	CCS	CCS	CCS
	30%	CCS	CCS	CCS	CCS

*ccs= clear, colorless solution, Ppt=precipitate

7.1.4 Solution Stability Studies

Table 25 Solution Stability Studies of 10%, 20% TBAat25^{0}C

S. No	Parameters	10% TBA Stored at 25^{0}C						20% TBAStored at25^{0}C					
		Time (hours)						Time (hours)					
		0	3	6	9	12	24	0	3	6	9	12	24
1	Description	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
2	pH	2.8	2.6	2.5	2.5	2.3	2.3	2.9	2.8	2.7	2.5	2.5	2.2
3	Assay (%)	104.7	97.6	89.2	85.6	80.2	76.3	104.6	100.5	99.4	95.7	93.1	88.9
4	Related substance												
	SingleMax(%)	1.87	10.01	16.67	20.08	22.54	30.32	1.37	5.42	8.71	10.9	12.3	18.49
	Total Imp (%)	2.04	10.96	19.12	23.58	27.01	39.6	1.51	5.93	9.70	12.3	14.0	22.0

Table 26 Solution Stability Studies of 30%, 40% TBAat25^{0}C

S. No	Parameters	30% TBA Stored at 25^{0}C						40% TBAstored at25^{0}C					
		Time (hours)						Time (hours)					
		0	3	6	9	12	24	0	3	6	9	12	24
1	Description	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
2	pH	3.1	3.0	2.9	2.9	2.8	2.6	3.3	3.2	3.0	2.9	2.7	2.7
3	Assay (%)	105.2	104	103.2	102.1	101.3	100	106	105	103	102	101.5	100
4	Related substance												
	Single Max(%)	0.93	2.47	3.15	3.87	4.66	6.86	0.95	1.56	3.12	3.98	4.75	6.50
	Total Imp (%)	1.05	2.71	3.48	4.29	5.17	7.73	1.12	2.77	3.48	4.79	5.67	7.57

Table 27 Solution Stability Studies of 10%, 20% TBA at 2-8^{0}C

S. No	Parameters	10% TBAStored at 2 - 8^{0}C						20% TBAstored at2 - 8^{0}C					
		Time (hours)						Time (hours)					
		0	3	6	12	18	24	0	3	6	12	18	24
1	Description	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
2	pH	2.7	2.6	2.5	2.5	2.3	2.3	2.9	2.8	2.7	2.6	2.6	2.4
3	Assay (%)	102.7	98.4	92.8	87.6	84.2	81	104.2	103.2	101.8	100.5	99.6	94.2
4	Related substance												
	Single Max(%)	1.26	1.94	2.85	4.62	6.23	8.71	0.10	0.27	0.64	0.97	1.27	1.94
	Total Imp (%)	1.48	2.12	3.02	5.21	6.81	9.68	0.24	0.59	1.15	1.54	1.98	2.58

Table 28Solution Stability Studies of 30%, 40% TBA at 2-8^{0}C

S. No	Parameters	30% TBAStored at 2 - 8^{0}C						40% TBAstored at2 - 8^{0}C					
		Time (hours)						Time (hours)					
		0	3	6	12	18	24	0	3	6	12	18	24
1	Description	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear	Clear
2	Ph	3.066	3.064	3.05	3.04	3.022	3.019	3.19	3.19	3.19	3,18	3.18	3.18
3	Assay (%)	105.2	104	103.2	102.1	101.3	100	99.3	97.9	101.6	100.9	101.5	99.0
4	Related substance												
	Single Max(%)	0.16	0.18	0.20	0.27	0.29	0.68	0.18	0.24	0.28	0.48	0.64	0.94
	Total Imp (%)	0.23	0.24	0.50	0.59	0.71	0.80	0.26	0.59	0.61	0.72	1.12	1.62

7.2 COMPATIBILITY STUDY RESUTS

Table 29PVDF Filter Compatibility Study

S.no	Test parameter	Specification	Initial	PVDF Filter compatibility		
				2hrs	4hrs	6hrs
1	Description	CCS	CCS	CCS	CCS	CCS
2	Assay (%)	90-100	99.5	99.3	99.1	99.08
3	pH after reconstitution	2.5-3.5	3.1	3.15	3.21	3.25
4	Related substances					
	BND-IV impurity (%)	0.55	ND	ND	ND	ND
	Dihydroxy impurity (%)	0.5	ND	ND	ND	ND
	Monohydroxy impurity (%)	0.5	0.248	0.249	0.252	0.252
	IPAester impurity (%)	0.5	0.042	0.042	0.043	0.045
	Single max (%)	1.0	0.032	0.033	0.035	0.036
	Total impurities (%)	NTM 3	0.322	0.324	0.329	0.333

***CCS=Clear Colorless Solution, ND=Not Detected, BEND-VI = Bendamustine, IPA = Isopropyl Acetate**

Table 30Silicon Tubing Compatibility Study (Pharma Pure Silicon Tube)

S.no	Test parameter	Specification	Initial	Pharma Pure compatibility (storage at 2-8 ºC)		
				2hrs	4hrs	6hrs
1	Description	CCS	CCS	CCS	CCS	CCS
2	Assay (%)	90-110	99.5	99.47	99.42	99.4
3	pH after reconstitution	2.5-3.5	3.1	3.14	3.16	3.21
4	Related substances					
	BND-IV impurity (%)	0.55	ND	ND	ND	ND
	Dihydroxy impurity (%)	0.5	ND	ND	ND	ND
	Monohydroxy impurity (%)	0.5	0.248	0.248	0.252	0.254
	IPAester impurity (%)	0.5	0.042	0.046	0.050	0.052
	Single max (%)	1.0	0.032	0.036	0.038	0.042
	Total impurities (%)	NTM 3	0.322	0.33	0.34	0.348

***CCS=Clear Colorless Solution, ND=Not Detected, BEND-VI = Bendamustine, IPA = Isopropyl Acetate**

Table 31SS Vessel Compatibility Study

S.no	Test parameter	Specification	Initial	SS Vessel compatibility (storage at 2-8 ºC)		
				2hrs	4hrs	6hrs
1	Description	CCS	CCS	CCS	CCS	CCS
2	Assay (%)	90-110	99.5	99.48	99.39	99.32
3	pH after reconstitution	2.5-3.5	3.1	3.15	3.22	3.22
4	Related substances					
	BND-IV impurity (%)	0.55	ND	ND	ND	ND
	Dihydroxy impurity (%)	0.5	ND	ND	ND	ND
	Monohydroxy impurity (%)	0.5	0.248	0.252	0.262	0.262
	IPAester impurity (%)	0.5	0.042	0.048	0.058	0.058
	Single max (%)	1.0	0.032	0.036	0.050	0.050
	Total impurities (%)	NTM 3	0.322	0.336	0.351	0.37

***CCS=Clear Colorless Solution, ND=Not Detected, BEND-VI = Bendamustine, IPA = Isopropyl Acetate**

7.3 FORMULATION RESULTS

7.3.1 Lyophilization of F1 formulation

Trail 1

Table 32Freezing

Controlled parameters	Events			
	1	**2**	**3**	**4**
Shelf temperature (^{0}C)	-15	-15	-40	-40
Time (min)	30	60	60	120
Final freeze temperature (^{0}C)	-40			
Duration of freezing (hours)	4.5			

Table 33PrimaryDrying

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	-30	-30	-20	-20	0	0	20	20	30	30
Time (min)	90	30	45	30	30	75	30	300	120	390
Vacuum set point (MT)	100	100	100	100	100	100	100	100	100	100
Duration of primary drying (hours)	19									

Table 34SecondaryDrying

Controlled parameters	Events
	1
Shelf temperature (^{0}C)	40
Time (min)	120
Vacuum set point (MT)	100
Final drying temperature (^{0}C)	40
Duration of secondary drying (hours)	2

Trail 2

Table 35 Freezing

Controlled parameters	Events					
	1	**2**	**3**	**4**	**5**	**6**
Shelf temperature (^{0}C)	-15	-15	-30	-30	-45	-45
Time (min)	5	30	30	120	30	180
Final freeze temperature (^{0}C)	-45					
Duration of freezing (hours)	6.5					

Table 36Primary Drying

Controlled parameters	Events											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Shelf temperature (^{0}C)	-20	-20	-15	-15	-10	-10	0	0	15	15	30	30
Time (min)	90	90	30	480	30	360	30	120	30	180	60	120
Vacuum set point (MT)	150	150	150	150	150	150	50	50	50	50	50	50
Duration of primary drying (hours)	27											

Table 37Secondary Drying

Controlled parameters	Events	
	1	**2**
Shelf temperature (^{0}C)	40	40
Time (min)	30	480
Vacuum set point (MT)	50	50
Final drying temperature (^{0}C)	40	
Duration of secondary drying (hours)	8.5	

Trail 3

Table 38Freezing

Controlled parameters	Events							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
Shelf temperature (^{0}C)	-15	-15	-20	-20	-30	-30	-45	-45
Time (min)	5	30	30	30	30	120	30	180
Final freeze temperature (^{0}C)	-45							
Duration of freezing (hours)	7.58							

Table 39 Primary Drying

Controlled parameters	Events													
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
Shelf temperature (^{0}C)	-30	-30	-20	-20	-15	-15	-10	-10	0	0	20	20	30	30
Time (min)	30	60	120	360	120	480	60	360	60	180	60	120	120	480
Vacuum set point (MT)	150	150	150	150	150	150	150	150	150	150	100	100	50	50
Duration of primary drying (hours)	43.5													

Table 40Secondary Drying

Controlled parameters	Events
	1
Shelf temperature (^{0}C)	40
Time (min)	480
Vacuum set point (MT)	50
Final drying temperature (^{0}C)	40
Duration of secondary drying (hours)	8

7.3.2 Lyophilization F2 Formulation

Trail 1

Table 41Freezing

Controlled parameters	Events					
	1	**2**	**3**	**4**	**5**	**6**
Shelf temperature (^{0}C)	-15	-15	-30	-30	-45	-45
Time (min)	5	30	30	120	90	180
Final freeze temperature (^{0}C)	-45					
Duration of freezing (hours)	7.58					

Table 42Primary Drying

Controlled parameters	Events											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Shelf temperature (^{0}C)	-20	-20	-15	-15	-10	-10	0	0	15	15	30	30
Time (min)	90	90	60	480	60	360	90	120	30	180	60	120
Vacuum set point (MT)	150	150	150	150	150	150	50	50	50	50	50	50
Duration of primary drying (hours)	29											

Table 43Secondary Drying

Controlled parameters	Events	
	1	**2**
Shelf temperature (^{0}C)	40	40
Time (min)	180	480
Vacuum set point (MT)	50	50
Final drying temperature (^{0}C)	30	
Duration of secondary drying (hours)	11	

Trail 2

Table 44Freezing

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	5	5	-30	-30	-40	-40	-25	-25-	-40	-40
Time (min)	5	10	60	90	180	180	90	180	240	360
Final freezing temperature (^{0}C)	-40									
Duration of freezing(hours)	23.25									

Table 45 Primary Drying

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	-20	-20	-15	-15	0	0	25	25	35	35
Time (min)	90	160	90	180	380	360	240	240	180	180
Vacuum set point (MT)	100	100	100	100	100	100	100	100	100	100
Duration of primary drying (hours)	32.16									

Table 46 Secondary Drying

Controlled parameters	Events
	1
Shelf temperature (^{0}C)	40
Time (min)	850
Vacuum set point (MT)	50
Final drying temperature (^{0}C)	40
Duration of secondary drying (hours)	14.1

Trail 3

Table 47 Freezing

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	5	5	-30	-30	-45	-45	-20	-20	-45	-45
Time (min)	20	30	90	60	90	120	90	300	120	180
Final freezing temperature (^{0}C)	-45									
Duration of freezing(hrs)	18.8									

Table48 Primary Drying

Controlled parameters	Events															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Shelf temperature (^{0}C)	-35	-35	-30	-30	-25	-25	-20	-20	0	0	15	15	30	30	35	0
Time (min)	120	180	120	180	120	180	120	180	180	360	180	360	180	360	360	0
Vacuum set point (MT)	150	150	150	150	150	150	150	150	150	150	100	100	50	50	50	0
Duration of primary drying (hours)	53															

Table 49 Secondary Drying

Controlled parameters	Events
	1
Shelf temperature (^{0}C)	35
Time (min)	850
Vacuum set point (MT)	50
Final drying temperature (^{0}C)	30
Duration of secondary drying (hours)	14.1

7.3.3 Lyophilization F3 Formulation

Trail 1

Table 50Freezing

Controlled parameters	Events					
	1	**2**	**3**	**4**	**5**	**6**
Shelf temperature (^{0}C)	-15	-15	-30	-30	-45	-45
Time (min)	5	30	30	120	90	180
Final freeze temperature (^{0}C)	-45					
Duration of freezing (hours)	7.58					

Table 51Primary Drying

Controlled parameters	Events											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Shelf temperature (^{0}C)	-20	-20	-15	-15	-10	-10	0	0	15	15	30	30
Time (min)	90	90	60	480	60	360	90	120	30	180	60	120
Vacuum set point (MT)	150	150	150	150	150	150	50	50	50	50	50	50
Duration of primary drying (hours)	29											

Table 52Secondary Drying

Controlled parameters	Events	
	1	**2**
Shelf temperature (^{0}C)	40	40
Time (min)	180	480
Vacuum set point (MT)	50	50
Final drying temperature (^{0}C)	30	
Duration of secondary drying (hours)	11	

Trail 2

Table 53 Freezing

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	5	5	-30	-30	-40	-40	-25	-25-	-40	-40
Time (min)	5	10	60	90	180	180	90	180	240	360
Final freezing temperature (^{0}C)	-40									
Duration of freezing(hrs)	23.25									

Table 54 Primary Drying

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	-20	-20	-15	-15	0	0	25	25	35	35
Time (min)	90	160	90	180	380	360	240	240	180	180
Vacuum set point (MT)	100	100	100	100	100	100	100	100	100	100
Duration of primary drying (hours)	32.16									

Table 55 Secondary Drying

Controlled parameters	Events
	1
Shelf temperature (^{0}C)	40
Time (min)	850
Vacuum set point (MT)	50
Final drying temperature (^{0}C)	40
Duration of secondary drying (hours)	14.1

Trail 3

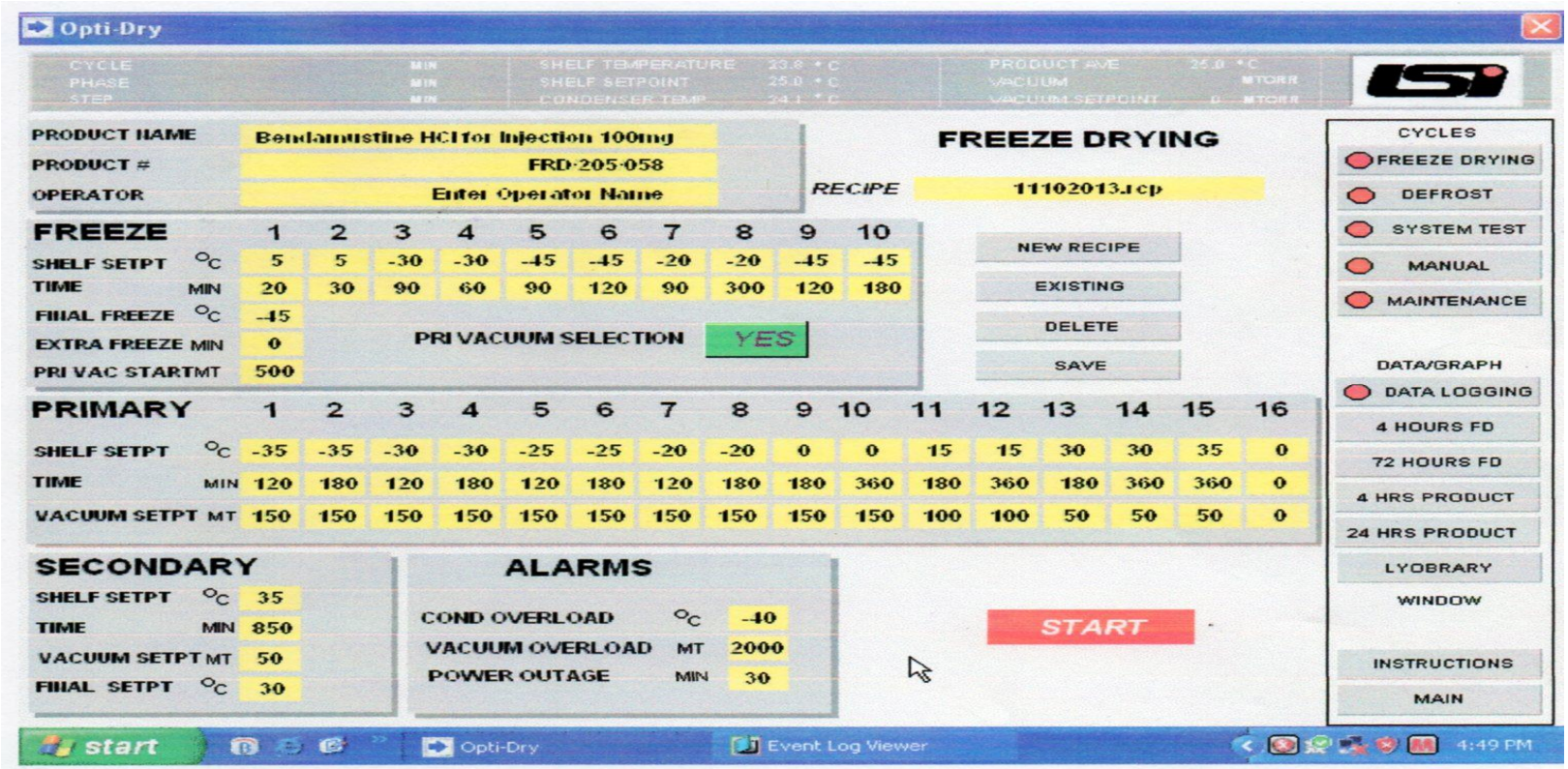


Fig 9 Lyophilization cycle of F3-tail 3 formulation

7.3.4 Lyophilization F4 Formulation

Trail 1

Table 56Freezing

Controlled parameters	Events					
	1	**2**	**3**	**4**	**5**	**6**
Shelf temperature (^{0}C)	-15	-15	-30	-30	-45	-45
Time (min)	5	30	30	120	90	180
Final freeze temperature (^{0}C)	-45					
Duration of freezing (hours)	7.58					

Table 57Primary Drying

Controlled parameters	Events											
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Shelf temperature (^{0}C)	-20	-20	-15	-15	-10	-10	0	0	15	15	30	30
Time (min)	90	90	60	480	60	360	90	120	30	180	60	120
Vacuum set point (MT)	150	150	150	150	150	150	50	50	50	50	50	50
Duration of primary drying(hours)	29											

Table 58Secondary Drying

Controlled parameters	Events	
	1	**2**
Shelf temperature (^{0}C)	40	40
Time (min)	180	480
Vacuum set point (MT)	50	50
Final drying temperature (^{0}C)	30	
Duration of secondary drying (hours)	11	

Trail 2

Table 59Freezing

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	5	5	-30	-30	-40	-40	-25	-25-	-40	-40
Time (min)	5	10	60	90	180	180	90	180	240	360
Final freezing temperature (^{0}C)	-40									
Duration of freezing(hrs)	23.25									

Table 60Primary Drying

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	-20	-20	-15	-15	0	0	25	25	35	35
Time (min)	90	160	90	180	380	360	240	240	180	180
Vacuum set point (MT)	100	100	100	100	100	100	100	100	100	100
Duration of primary drying (hours)	35.16									

Table 61Secondary Drying

Controlled parameters	Events
	1
Shelf temperature (^{0}C)	40
Time (min)	850
Vacuum set point (MT)	50
Final drying temperature (^{0}C)	40
Duration of secondary drying (hours)	14.1

Trail 3

Table 62 Freezing

Controlled parameters	Events									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
Shelf temperature (^{0}C)	5	5	-30	-30	-45	-45	-20	-20	-45	-45
Time (min)	20	30	90	60	90	120	90	300	120	180
Final freezing temperature (^{0}C)	-45									
Duration of freezing(hrs)	18.8									

Table 63 Primary Drying

Controlled parameters	Events															
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**	**13**	**14**	**15**	**16**
Shelf temperature (^{0}C)	-35	-35	-30	-30	-25	-25	-20	-20	0	0	15	15	30	30	35	0
Time (min)	120	180	120	180	120	180	120	180	180	360	180	360	180	360	360	0
Vacuum set point (MT)	150	150	150	150	150	150	150	150	150	150	100	100	50	50	50	0
Duration of primary drying (hours)	53															

Table 64 Secondary Drying

Controlled parameters	Events
	1
Shelf temperature (^{0}C)	35
Time (min)	850
Vacuum set point (MT)	50
Final drying temperature (^{0}C)	30
Duration of secondary drying (hours)	14.1

7.4 EVALUATION RESULTS

Table 65

S.NO	TEST	F 1			F 2			F 3			F 4		
		T 1	T 2	T 3	T 1	T 2	T 3	T1	T2	T 3	T 1	T 2	T 3
1	Cake appearance	S	S	S	G	G	G	G	G	G	G	G	G
2	Clarity of reconstituted solution	CCS	CCS	CCS	CCS	CCS	CCS	CCS	CCS	CCS	CCS	CCS	CCS
3	Reconstitution time	15 min	16 min	15 min	7min	2min	92 sec	5min	2min	32sec	4 min	1.5 min	60 sec
4	P^H after reconstitution	2.3	2.3	2.5	2.5	2.8	2.9	2.9	2.9	3.1	3.5	3.5	3.2
5	Water content (%w/w)	5.8	5	4.2	4	2	0.99	3.6	2	0.98	1	1.6	1
6	Assay (%)	50	55	50	70	90	96.5	85	92.5	99.5	96	91.8	97
7	Related substances (%)	3.2	3.2	3.4	3	2.1	0.25	2.5	1.8	0.248	2.5	0.99	2.5

*** T = Trail, CCS= Clear colorless solution, S= shrinkage, G=good**

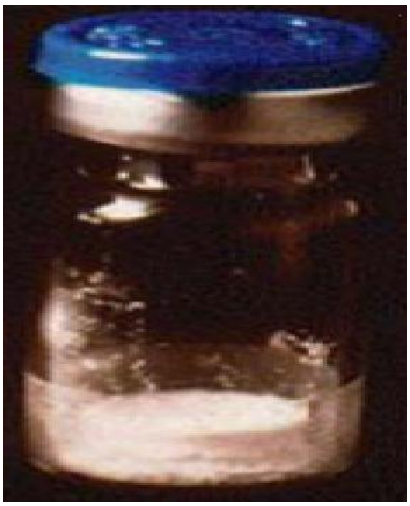


Fig 10Shrinkage Cake

Fig 11Good cake

Fig 12Cake of optimized formulation

7.5 ASSAY CHROMATOGRAM FOR STANDARD AND OPTIMIZED FORMULA

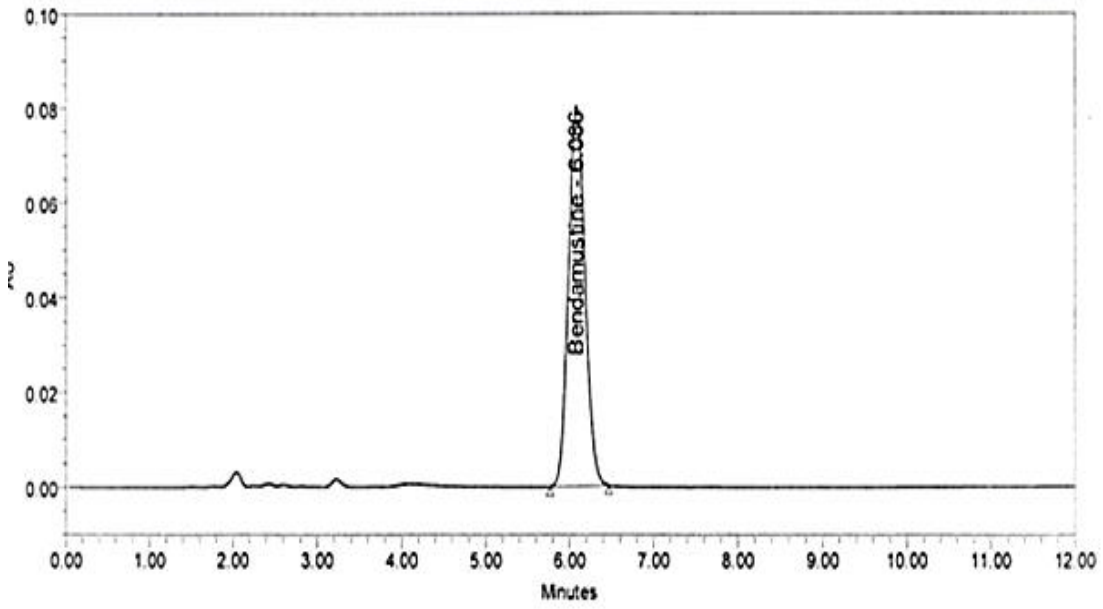


Fig 13 Chromatogram of standard

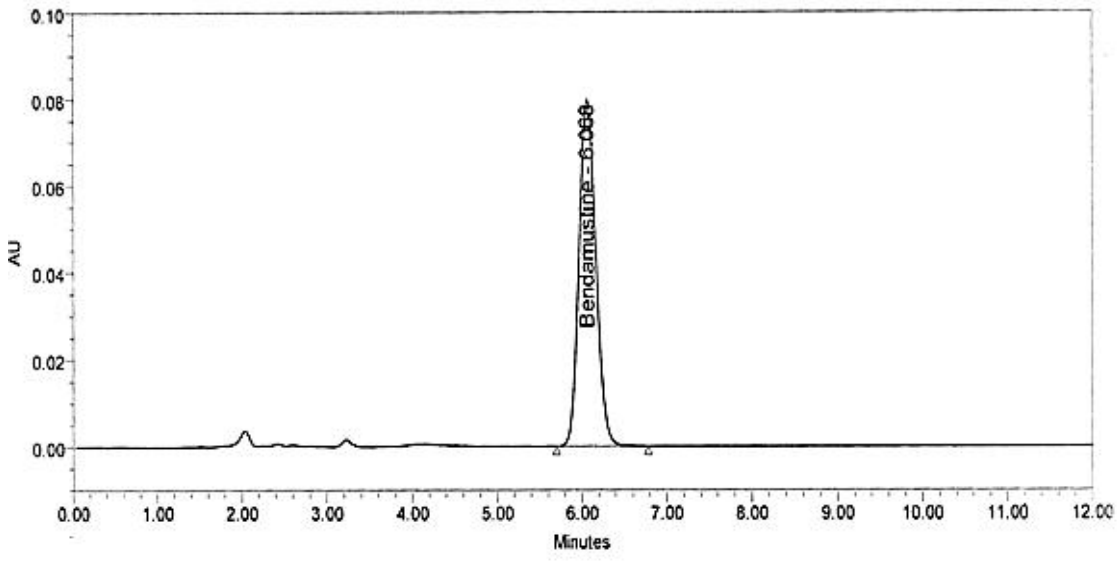


Fig 14Chromatogram of Optimized formulation (F3 –Trail 3)

7.6 DILUENT COMPATIBILITY STUDIES OF AN OPTIMIZED FORMULATION (F3-trail 3)

Table66

S.No	Parameters	Diluent Compatibility at25^{0}C		Diluent Compatibility at 2-8^{0}C	
		Initial	**3hr**	**24hrs**	**48hr**
1	Description	CCS	CCS	CCS	CCS
2	Assay (%)	100.2	100.0	100.1	100.2
3	Related Substances				
I	BND-VI Impurity (%)	ND	ND	ND	ND
Ii	Dihyroxy Impurity (%)	ND	ND	ND	ND
Iii	Monohydroxy Impurity (%)	0.232	0.235	0.248	0.242
Iv	Monochloro Impurity (%)	0.040	0.038	0.039	0.041
V	IPA Ester Impurity (%)	0.030	0.028	0.021	0.022
Vi	Single Max (%)	0.240	0.249	0.240	0.239
	Total (%)	0.542	0.550	0.548	0.544
4	Osmolality (m.osm)	292	292	292	292

*** CCS=Clear Colorless Solution, ND=Not Detected, BEND-VI = Bendamustine, IPA = Isopropyl Acetate**

7.7 INNOVATOR PRODUCT DETAILS

Table 67 Physical Parameters of Innovator Product

Water content	1.0%
pH	2.90
Reconstitution time	45 Sec
Assay	99.3%
Related substances	0.305%
Osmolality	295 m.osm

7.8 COMPARATIVE STUDY

Table 68 Comparison of Optimized Formulation with Innovator's Formulation (Treanda)

S.No	Parameters	Specifications	Innovator (Treanda)	Optimized formulation (F3-TRAIL 3)
1	Description	White to half white lyophilized cake or powder filled in 50 ml vial	White to half white lyophilized cake or powder filled in 50 ml vial	White to halfwhite lyophilized cake or powderfilled in 50 ml vial
2	pH after reconstitution	2.50-3.50	2.90	3.1
3	Reconstitution time	NMT 5 min	45 sec	32 sec
4	Water content (%w/w)	NMT 3	1.0	0.98
5	Osmolality (m.osm)	290-310	295	295
6	Assay (%)	90-110	99.3	99.5
7	Related substances			
i	BND-IV impurity (%)	0.55	ND	ND
ii	Dihydroxy impurity (%)	0.5	ND	ND
iii	Monohydroxy impurity (%)	0.5	0.253	0.248
iv	IPAester impurity (%)	0.5	0.030	0.042
v	Single max (%)	1.0	0.022	0.032
	Total (%)	3.0	0.305	0.322

***NMT = Not More Than, ND = Not Detected, BEND-VI = Bendamustine, IPA = Isopropyl Acetate**

7.9 STABILITY STUDIES AS PER ICH GUIDELINES

Table 69 Accelerated Stability Study

S.No	Test parameter	Specifications	Initial	40^0C/75 % RH	
				1st Month	2nd Month
1	Description	White to off white lyophilized powder	White to off white lyophilized powder	White to off white lyophilized powder	White to off white lyophilized powder
2	Assay	90-110	99.5	99.5	99.4
3	Water content(%w/w)	NTM 3	0.98	0.98	0.99
4	P^H after reconstitution	2.5-3.5	3.1	3.1	3.0
5	Reconstitution time	NTM 5 min	32 sec	32 sec	33 sec
6	Clarity of reconstitution solution	CSS	CSS	CSS	CSS
7	Osmolality (m.osm)	290-310	290	290	290
8	Related substance				
	BND-IV impurity (%)	0.55	ND	ND	ND
	Dihydroxy impurity (%)	0.5	ND	ND	ND
	Monohydroxy impurity (%)	0.5	0.248	0.248	0.249
	IPAester impurity (%)	0.5	0.042	0.042	0.042
	Single max (%)	1.0	0.032	0.032	0.032
	Total (%)	NMT 3	0.322	0.324	0.324

Table 70Stability Data of 3rd Month under Accelerated and Long Term Storage Conditions

S.No	Test parameter	Specifications	40^0C/75 % RH	25^0C/60 % RH
			3rd Month	3rd Month
1	Description	White to off white lyophilized powder	White to off white lyophilized powder	White to off white lyophilized powder
2	Assay	90-110	99.1	99.2
	Water content(%w/w)	NTM 3	1.03	0.98
4	P^H after reconstitution	2.5-3.5	3.1	3.1
5	Reconstitution time	NTM 5 min	35 sec	34 sec
6	Clarity of reconstitution solution	CSS	CCS	CCS
7	Osmolality (m.osm)	290-310	290	290
8	Related substance			
	BND-IV impurity (%)	0.55	ND	ND
	Dihydroxy impurity (%)	0.5	ND	ND
	Monohydroxy impurity (%)	0.5	0.252	0.249
	IPAester impurity (%)	0.5	0.045	0.044
	Single max (%)	1.0	0.034	0.034
	Total (%)	NMT 3	0.331	0.327

7.10 PHOTOSTABILITY STUDY

Table 71

S.No	Test parameter	Initial	Control (AL wrapped)	Secondary peak	Primary peak (delabelled)	Primary peak (labelled)
1	Description	White freeze dried powder	White freeze dried powder	White freeze dried powder	Pale yellow colored freeze dried powder	White freeze dried powder
2	Assay (%)	99.5	99.5	99.5	98.6	99.4
3	Water content (%w/w)	0.98	0.98	0.98	0.101	0.99
4	P^H after reconstitution	3.1	3.1	3.1	3.3	3.2
5	Reconstitution time	32 sec	32 sec	32 sec	44 sec	38 sec
6	Clarity of reconstituted solution	Clear , color less solution	Clear , color less solution	Clear , color less solution	Clear , color less solution	Clear , color less solution
7	Osmolality (m.osm)	290	290	290	290	290
8	Related substances (%)	0.322	0.322	0.322	0.456	0.35

8 DISCUSSION

8.1 PREFORMULATION STUDYS

8.1.1 Standard graph

Standard graph was plotted between the peak area and concentration of bendamustine HCL. The correlation co-efficient R^2 wasfound to be 0.9994 (fig 9).

8.1.2 Description

The received sample of API after visual observation it was found to show the white to off-white color, it is acceptable according to COA (certificate of analysis)

8.1.3 Saturation Solubility studies

a) In water

The solubility of Bendamustine hydrochloride was found to be 60mg/ml.

b) In water and alcohol

Bendamustine hydrochloride solubility depends on temperature and amount of alcohol in aqueous solution. For the alcohol tested the solubility of bendamustine HCL increased as the concentration of alcohol increased.

Alcohols varied in their effect on solubility without washing to be bound to any particular theory. Smaller alcohols such as methanol and ethanol have less of an effect on solubility as compared with larger alcohols (TBA and n-butanol)

However, the structure of alcohol is also important. For e.g.: n-propanol was found to be better than iso-propanol in preventing precipitation in this system. The two alcohols with the greater effect as solubility were n-propanol and TBA.

8.1.4 Solution stability studies

A) Solution stability studies of 10%, 20%, 30%, 40% TBA at 25^0C

From theresults(tables 25 and 26), the following results were observed:

Description: The test samples are clear, colorless solutions at the end of study.

pH: The pH of the samples decreased when increasing the hold time in different concentration of TBA.

Assay: Significant decrease amount of drug was observed when increasing the hold time at 25^0**C**

Related substances: Significant increase in related substances was observed when increasing the hold time at 25^0**C**

Conclusion: Bendamustine Hydrochloride formulation is unstable in solution form in all the concentrations of TBA when stored at 25^0**C**

Increasing concentrations of TBA, decreasing the degradation was observed.

B) Solution stability studies of 10%, 20%, 30%, 40% TBA at 2-8^0C

From the results (tables 27 and 28), the following results were observed:

Description: The test samples were clear, colorless solutions at the end of study.

pH: The pH of the samples decreased when increasing the hold time in different concentration of TBA.

Assay: Significant decrease of amount of drug was observed when increasing the hold time at 2-8^0Cin concentration of 10%TBA.

There is no significant decrease of amount of drug was observedwhen increasing the hold time at 2-8^{0}Cin concentrations of 20%, 30%, 40% TBA.

Related substances: Significant increase in related substances was observed when increasing the hold time at 2-8^{0}Cin concentrations of 10%TBA.

There is no significantincrease in related substances was observedwhen increasing the hold time at 2-8^{0}Cin concentrations of 20%, 30%, 40% TBA.

Conclusion: Bendamustine Hydrochloride formulation is unstable in solution form in concentrations of 10% TBA when stored at 2-8^{0}C, whereas it is stable in concentrations of 20%, 30%, 40% TBA when stored at 2-8^{0}C.

From the results based on solution stability studies, the concentrations of 20%, 30%, 40% TBA v/v were used further in the experiment.

8.2 COMPATIBILITY STUDIES WITH PROCESS COMPONENTS

8.2.1 Filter Compatibility Study

From the result, it was found that the assay,pH after reconstitution and related substances were present within the specification limits (Table 29). Hence, it can be concluded that PVDF filter can be used for the purpose of the filtration during the experimental work.

8.2.2 Pharma Pure Tube Compatibility Study

From the results, it was found that the assay,pH after reconstitution and related substances were present within the specification limits(Table 30). Hence, it can be concluded that Pharma tube silicon tubing can be used as processing aid during filtration and filling operation for transfer of liquid in pharmaceutical manufacturing during the experimental work.

8.2.3 SS Vessel Compatibility Study

From the results, it was found that the assay,pH after reconstitution and related substances were present within the specification limits (Table 31). Hence SS Vessel was found to be compatible with the drug solution throughout the study period and therefore it was used for holding the drug solution before loading in to the lyophilizer.

8.3 FORMULATION RESULTS

8.3.1 Lyophilization of F1 formulation

Trail 1

From the results, F1- Trail1 Formulation was formulated as mentioned in Table 10. The F1-Trail 1 formulation was then subjected to lyophilization as per the conditions shown in Tables 32–34.Initial freezing was conducted in four events for about 4.5 hrs with a final freezing temperature of -40^0C. It was then followed by primary drying in 10 events for 19 hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in one event for 2 hrs.

It was observed that after lyophilization, the cake shrinkage making it to collapse. This leads to poor rehydration. The possible reason could be due to

- Sudden decrease in freezing temperature
- Low solubility of bendamustine hydrochloride in water.

Therefore, further optimization was done in the lyophilization process to improve the cake characteristics.

Trail 2

From the results, F1-Trail 2 Formulation was formulated as mentioned in the Table 10. The F1- Trail 2 formulation was then subjected to lyophilization as per the conditions shown in Tables 35-37. Initial freezing was conducted in 6 events for about 6.5 hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 12 events for 27hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in 2events for 8.5 hrs.

It was observed that after lyophilization, the cake shrinkage making it to collapse. This leads to poor rehydration. The possible reason could be due to sudden decrease in freezing temperature, Low solubility of bendamustine hydrochloride in water

Therefore, further optimization was done in the lyophilization process to improve the cake

Trail 3

From the Results, F1- Trail 3 Formulation was formulated as mentioned inTable 10.The F1- Trail 3 formulation was then subjected to lyophilization as per the conditions shown in Tables 38-40. Initial freezing was conducted in 8 events for about 7.5 hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 14events for 43.5hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in one event for 8 hrs.

It was observed that after lyophilization, the cake shrinkage making it to collapse. This leads to poor rehydration. The possible reason could be due to

- Primary drying is not completed
- Low solubility of bendamustine hydrochloride in water.

Therefore, further optimization was done in the lyophilization process to improve the cake characteristics.

8.3.2 FORMULATION 2

Trail 1

From the results, F 2- Trail 1 formulations was formulated as mentioned in the Table 10. The F2-Trail 1 formulation was then subjected to lyophilization as per the conditions shown in Tables 41-43 .Initial freezing was conducted in 6 events for about 7.58 hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 12 events for 29hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in one event for 11 hrs.

After lyophilization, the cake was found to be good but water content and reconstitution time obtained were not satisfactory. The possible reason could be due to Insufficient freezing, Insufficient duration of cycle.

Therefore, further optimization was doneby employing annealing technique in freezing process and further prolonging the cycle duration.

Trail 2

From the results, F 2- Trail 2 formulations were formulated as mentioned in the method using the formula given in Table 10.The F2-Trail 2 formulation were then subjected to lyophilization as per the conditions shown in Tables 44-46. Initial freezing was conducted in 10 events for about 23.25hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 10 events for 32.16 hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in 1event for 14.1 hrs.

After lyophilization, the water content of the formulations was found to be within the prescribed limit but reconstitution time and residual solvents were out of limits. The possible reason could be due to insufficient duration of cycle, insufficient drying temperature and time.

Trail 3

From the results, F2-Trail 3 formulations were formulated as mentioned in the method using the formula given in Table 10. The formulations were then subjected to lyophilization as per the conditions shown in the table 47-49. Initial freezing was conducted in 10 events for about 18.8hrs with a final freezing temperature of -45^{0}C. It was then followed by primary drying in 15 events for 53hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in 1event for 14.1 hrs.

After lyophilization, all the parameters of the formulations i.e.,Clarity, Reconstitution time, pH after reconstitution, water content, Assay and Related substances was found to be good . Through the reconstitution time was decrease than trai 2, further to decrease the reconstitution time the concentration of TBA: WFI was chaged and next trail were performed.

8.3.3 FAROMULATION 3

Trail 1

From the results, F 3- Trail 1 formulations were formulated as mentioned in the method using the formula given in Table 10. The F3- Trail 1 formulation were then subjected to lyophilization as per the conditions shown in Tables 50-52.Initial freezing was conducted in 6 events for about 7.58 hrs with a final freezing temperature of -45^{0}C. It was then followed by primary drying in 12 events for 29hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in one event for 11 hrs.

After lyophilization, the cake was found to be good but water content and reconstitution time obtained were not satisfactory. The possible reason could be due to Insufficient freezing, Insufficient duration of cycle.

Therefore, further optimization was doneby employing annealing technique in freezing process and further prolonging the cycle duration.

Trail 2

From the results, F3-Trail 2 formulations were formulated as mentioned in the method using the formula given in Table 10.The F3-taril 2 formulation were then subjected to lyophilization as per the conditions shown in Tables 53-55. Initial freezing was conducted in 10 events for about 23.25hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 10 events for 32.16 hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in 1event for 14.1 hrs.

After lyophilization, the water content of the formulations was found to be within the prescribed limit but reconstitution time and residual solvents were out of limits. The possible reason could be due to insufficient duration of cycle, insufficient drying temperature and time.

Trail 3

From the results, F3-Trail 3formulationswere formulated as mentioned in Table 10. The formulations were then subjected to lyophilization as per the conditions shown in the fig 9. Initial freezing was conducted in 10 events for about 18.8hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 15 events for 51.3hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in 1event for 14.1 hrs.

The reconstitution was 32 sec which was found satisfactory with change in TBA: WFI and cycle changed

Further to see the improvement formulation 4 with TBA: WFI (80:120) was prepared

8.3.4 FAROMULATION 4

Trail 1

From the results, F4-Trail 1 formulations were formulated as mentioned in Table 10. The F4-Trail 1 formulation were then subjected to lyophilization as per the conditions shown in Tables 56-58 .Initial freezing was conducted in 6 events for about 7.58 hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 12 events for 29hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in one event for 11 hrs.

After lyophilization, the cake was found to be good but water content and reconstitution time obtained were not satisfactory. The possible reason could be due to Insufficient freezing, Insufficient duration of cycle.

Therefore, further optimization was doneby employing annealing technique in freezing process and further prolonging the cycle duration.

Trail 2

From the results, F4-Trail 2 formulations were formulated as mentioned in Table 10.The F4-Trail 2 formulation were then subjected to lyophilization as per the conditions shown in Tables 59-61. Initial freezing was conducted in 10 events for about 23.25hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 10 events for 32.16 hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in 1event for 14.1 hrs.

After lyophilization, the water content of the formulations was found to be within the prescribed limit but reconstitution time and residual solvents were out of limits. The possible reason could be due to insufficient duration of cycle, insufficient drying temperature and time.

Trail 3

From the results, F4-Trail 3 formulations were formulated as mentioned in Table 10. The formulations were then subjected to lyophilization as per the conditions shown in the tables 62-64. Initial freezing was conducted in 10 events for about 18.8hrs with a final freezing temperature of -45^0C. It was then followed by primary drying in 15 events for 53 hrs by applying vacuum throughout the cycle. Finally, the secondary drying was carried out in 1event for 14.1 hrs.

This formulation also shows same reconstitution time as formulation formulation 3 trail 3

Depending upon the concentration of solvents formulation 3-trail 3 was taken as optimized formulation

8.4 EVALUATION RESULTS

From the data illustrated in the Table 65, it was observed that the clarity of all the formulations was clear and pH (2.5-3.5) was within the prescribed limits. In F1-Trail 1, Trail 2, Trail 3 formulations shrinkage of the cake was observed as shown in the fig 10, but from formulation F2-trail 1 to F4- trail 3, it was found to be good as shown in the fig 11. The water content of the formulations F3Trai 1 to F4-trai 3 was within the prescribed limits (NMT 3%). From formulations F2 –trail 2 to F4- trail 3, the presence of related substances (2.5%) was found to be within the limits. The assay (90-110%) of the formulations F2-tail 3, F3-trail 3, F4- trail 3 was found to be better. Among all the formulations the reconstitution of the formulation F3-trail 3 was found to be the best.

Therefore, it can be evidently concluded that the formulation F3-Trail3 was the best formulation (Fig 12). Chromatogram of assay of an optimized formulation F3-Trail3 was illustrated in the figure 14.

8.5 DILUENT COMPATIBILITY STUDIES OF AN OPTIMIZED FORMULATION (F3-trail 3)

From the results Table 66, it was observed that the reconstituted lyophilized product was compatible with 0.9% sodium chloride solution. No incompatibility was observed in the diluent and formulation. The drug solution was stable for 3hrs when stored at 25^0C and for 48 hrs at 2-8^0C.

8.6 COMPARISION OF OPTIMIZED FORMULATION WITH INNOVATOR'S FORMULATION (TREANDA)

The test parameters like pH after reconstitution, Reconstitution time, Water content, Assay, Osmolality and Related substances of the optimized formulation was compared with innovators drug product. From the results (table 68) it was found that the formulation was similar with that of innovator.

8.7 STABILITY STUDIES AS PER ICH GUIDELINES

ACCELERATED STABILITY STUDY

From the results Tables 69 and 70, The accelerated and long term stability study results confirms that the test parameters like pH after reconstitution, Reconstitution time,Water content, Assay, Osmolality and Related substances were found to be within the specification limits. So, it can be concluded that the lyophilized drug was stable at accelerated and long term storage conditions of 40°C/75 % RH and 25°C/60 % RH respectively for a period of 3 months.

8.8 PHOTOSTABILITY STUDY

From the results Table 71, the photo stability study results confirm that the test parameters like pH after reconstitution, Reconstitution time, Water content, Assay, Osmolality and Related substances were found to be within the specification limit. Hence the photo stability study on the drug product confirmed that the product was stable when exposed to the light in its primary packing.

SUMMARY & CONCLUSION

Bendamustine Hydrochloride is unstable in the solution form. So, it cannot be formulated as a liquid dosage form. In order to improve its stability, the drug product should be dried and formulated as a solid product. Even though there are several drying techniques to dry a product, lyophilization technique was preferred for drying this product because it involves the drying of the product at low temperature and low pressure. As Bendamustine Hydrochloride is thermolabile in nature and cannot withstand elevated temperatures, this technique is the best choice to increase its stability.

Preformulation studies were done by conducting solution stability studies at different concentrations of TBA (10%, 20%, 30%, and 40%) at 25°C and 2-8°C upto 24hrs. From these studies it was found that the drug solution was unstable in all concentrations of TBA at 25°C and in 10%TBA at 2-8°C. So, further studies were carried out with 20%, 30% and 40% TBA at 2-8°C.

Initially an injectable dosage form of the drug product was developed and the compatibility of this drug solution with various process components was studied. The drug was found tobe compatible with the used materials (SS Vessel, pharmapure silicon tube, PVDF filter).

The thermal characteristics of the product were studied and based on that different lyophilization cycles have been developed by varying temperature, pressure and duration of the cycle. The final temperatures set for the optimized formulation was able to withstand freeze thaw cycles without effecting the quality of the product. The freezing, primary drying and secondary drying temperatures used were -45°C, 35°C and 35°C respectively, the pressure during the process was set as 150-50mTorr and the total run time of the cycle was 84.2 hours.

The formed lyophilized cake was evaluated for its appearance, water content, assay, reconstitution time, p^H and related substances. All these parameters

were found to be within the specifications thus confirming that the developed lyophilization cycle was proved to be satisfactory.

The reconstitution stability studies showed that the developed lyophilized product can be reconstituted with the innovator recommended diluents such as 0.9% sodium chloride injection within a specified time.

The results of the optimized formulation was found to be good as that of innovator.

The photo stability study on the drug product confirmed that the product was stable when exposed to the light in its primary packing.

CONCLUSION

The lyophilized technique proved to be an advantage for development of stable injectable dosage form of Bendamustine Hydrochloride as the moisture content of the formulation was greatly reduced to as low as 0.98% (w/w) thus enhancing the stability of the product.However, further work in this field of study require in order to make the drug available in the market.

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